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CARL VON VOIT

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Carl Voit was born in Amberg, Bavaria, October 31, 1831. He was the son of a well-known architect, August Voit, who later in 1854 designed the Crystal Palace in Munich, the locale of his son's life work. After completing his work in the gymnasium, Carl applied himself in 1848 to the study of medicine at the University of Munich. In 1851 to 1852 he attended the University of Würzburg, which enjoyed the highest scholastic reputation in Germany at that time. Here he listened to lectures by such renowned men as Köllicker, Leydig, Virchow, Skanzoni and Scherer. In 1852 he was back at Munich, where in the meantime Liebig and Pfeufer had been called, initiating a new scientific life in the Hochschule. Completing his medical course in 1854, Voit continued his studies in the natural sciences under the physicist Jolly, the zoölogist Siebold, the anatomist and physiologist Bischoff and the chemist Liebig.

On the advice of Liebig, in 1855 Voit went to Göttingen, where he spent a most inspiring year in Wöhler's laboratory. Here he made a special study of benzoyl compounds and, under Listing, acquired an unusual knowledge of physiological optics.

In 1856 Voit was appointed Privat-dozent in the Physiological Institute of Munich, becoming an assistant to Pettenkofer, professor of medical chemistry. Here he published his first scientific work, a paper entitled: "Untersuchungen von Bodenarten zur Entscheidung pflanzen physiologischer Fragen." This was followed shortly by a paper "Über die Aufnahme von Quecksilber und seiner Verbindungen in den

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Körper," and by the noteworthy paper, published with Bischoff, "Die Gesetze der Ernährung des Fleischfressers," in 1860.

In 1859 he declined a call to Tübingen. On the death of Harless, professor of physiology at Munich, in 1863, Voit succeeded to his chair and also to the directorship of the Physiological Institute, positions that he held until his death on January 31, 1908. His renown brought him many honors, including election to membership in the Academy of Sciences in 1865. In the same year, he with Buhl, Pettenkofer and Radlkofer founded the Zeitschrift für Biologie.

At 55, when Lusk first knew Voit, he was "alert, with a quick walk, of quiet, dignified, courteous bearing." He was as simple as a German of the old school. He never attended scientific meetings except those of the local scientific society, which were social in nature. "Only through his Zeitschrift für Biologie did he rub shoulders with the world." He attracted many students and possessed the quality of holding their affection and loyalty. If judged only by the performance of those who received their training in his laboratory, his influence on the development of the science of nutrition was enormous. Among his students are numbered Rubner, Prausnitz, Atwater, Lusk, Y. Henderson, E. Voit, Cathcart, Max Cremer, Otto Frank, who succeeded him as director of the Physiological Institute, and many others of note.

Carl Voit is often referred to as the founder of metabolic research. While Liebig opened a new era in the science of nutrition—the quantitative and therefore the scientific era—he was essentially a chemist, not a biologist. He was a great experimentalist but his theories of nutrition and metabolism were not based upon experiment: in fact, he never performed an animal experiment. On the contrary, Voit was well grounded in biology, as well as the other natural sciences, and developed a refined experimental technic for the study of animal physiology. While Liebig elaborated chemical methods for the searching analysis of animal tissues and excretions, Voit applied these methods to well-controlled

metabolism studies of the income and outgo of animals and of men. With painstaking care and an attention to experimental details that excited the admiration of his colleagues, he elaborated a methodology in metabolic research that was a model for others to follow. As Lusk has said, *"Genauigkeit* was the watchword of his laboratory." Supremely critical of the work of others, he was consistent in being as critical of his own work. Any uncertainty concerning the accuracy of a method of analysis, an analytical result, or an excretory collection was sufficient basis for discarding an experiment.

At the very beginning of his career, he believed that only by rigid methods of experimental control could the principles of animal metabolism be uncovered. Because of the inspiration he received from Wöhler, Liebig, Bischoff and Pettenkofer, and the interest he felt in the well-known work of Bidder and Schmidt at Dorpat, he turned first to the determination of the metabolism of protein, by following the income and outgo of nitrogen.

With the exception of the work of Bidder and Schmidt, previous experiments in this field had yielded little useful information, because, no matter what the protein intake might be, nitrogen equilibrium could not be demonstrated. There always remained a bothersome 'nitrogen deficit' that was quite commonly ascribed to a metabolism of gaseous nitrogen. It was presumed that some volatile substance containing nitrogen, such as ammonia, was expired through the lungs, and even that atmospheric nitrogen entered into the animal economy. Even his master, Bischoff, had been unable to attain nitrogen equilibrium in animal experiments.

With Bischoff, and with his new methods of experimentation, Voit immediately obtained results of the greatest importance. The troublesome 'nitrogen deficit' disappeared. In experiments on dogs, and later in a 124-day metabolism experiment on a pigeon, it was shown that on adequate feeding the nitrogen of the solid and liquid excreta equals the nitrogen consumed, and that this condition of nitrogen equilibrium is always obtained with adult animals on constant

feeding inside very wide limits of nitrogen intake. The 'nitrogen deficit' was evidently the child of inaccurate analytical methods.

This finding, together with the later determination that the losses of nitrogen through hair growth, epidermis and sweat secretion is generally negligible, enormously simplified the technic of protein studies, which consequently are concerned only with the nitrogen content of food, feces and urine, except for special problems. The importance of the establishment of a condition of nitrogen equilibrium to studies of the effects of any factor on protein metabolism is obvious.

The protein metabolism studies of Bischoff and Voit on dogs uncovered many other important facts. They showed that in fasting there is always a considerable nitrogen loss; that the consumption of meat leads to an equilibrium of intake and outgo of nitrogen, which may be reestablished at any higher meat intake after the lapse of a sufficient adjustment period; that the giving of fat or carbohydrate to an animal in nitrogen equilibrium decreased the loss of nitrogen. In particular, it was shown that muscular work does not increase the nitrogen output of a dog receiving an adequate intake of non-nitrogenous nutrients, a finding that was confirmed later on human subjects by Ranke, a pupil of Voit, and later still by Voit himself. These studies, together with the contemporary observations of Fick and Wislicenus in 1865 made during their ascent of the Faulhorn, and particularly the more complete observations of N. Zuntz during his ascent of the much higher Monte Rosa in 1906, sounded the death knell for the theory of Liebig that the energy for muscular work could be derived only from the decomposition of muscle tissue itself. Liebig based his whole theory of nutrition upon the unique value of dietary protein in replacing these muscle losses, as well as the losses of glandular substance during the process of secretion.

With Bischoff, Voit developed the conception of feces formation that is valid today. They observed that feces were formed during fasting and that during nutrition on highly

digestible diets the feces formed were not proportional in amount to the food consumed. Hence, they consist largely of the unabsorbed residues of the digestive juices.

From studies on the metabolism of protein, Voit's interest naturally turned to the metabolism of carbohydrates and fats. But such an extension of his program required the construction of a respiration apparatus for the determination of the outgo of carbon and the income of oxygen. It was Pettenkofer's great contribution to design a chamber large enough to accommodate a man, equipped to permit continuous observations of the gaseous exchange over many hours, and ventilated by the open circuit method so that such protracted confinement would not become irksome. The apparatus was a great improvement over those theretofore used, and the life-long friendship of Pettenkofer and Voit gave to science a series of important facts and principles established by the use of this instrument of research. Later, with a modified respiration chamber, Voit continued on laboratory animals his investigations of the total metabolism. The computations made from the recorded measurements of the income and outgo of nitrogen and carbon and the consumption of oxygen were expressed not in calories, but in the amounts of protein, carbohydrate and fat stored and oxidized in metabolism.

Pettenkofer and Voit found that in starvation only protein and fat are burned; that during work more fat is oxidized, but that during rest fat is readily deposited in the body; that carbohydrates are completely burned no matter how much is given, in this manner protecting the stores of fat. They believed that the metabolizability of the nutrients in the body is not proportional to their combustibility outside the body. Thus, proteins are the most easily metabolizable but are combustible with difficulty in vitro. On the contrary, fats are difficultly metabolizable, but most readily combustible in the laboratory. Carbohydrates are intermediate in both respects.

While Voit's researches with Pettenkofer were not calorimetric studies in the modern conception of the term they afforded the impetus for the development of the modern

theories of the metabolism of energy. Rubner served his apprenticeship under Voit and later at Marburg he continued his brilliant work in his own laboratory, establishing the validity in the animal body of the law of the conservation of energy, defining the replaceability of the nutrients in metabolism, illuminating the regulatory mechanism for the maintenance of the body temperature of homeotherms, confirming the surface area law of basal energy expenditures, and measuring the specific dynamic effects of the nutrients. All these epoch-making researches had their inception in the laboratory of Voit at Munich.

Early in their association, Pettenkofer and Voit concerned themselves with the metabolism of disease—diabetes, leucemia, anemia, fever. However, since at that time clinics and hospitals were not provided with respiration apparatus as they are today, they were perforce restricted to studies of the metabolism of nitrogen. Comparisons were made with healthy people on the same intake of food.

True to the traditions of Liebig, Voit did not confine himself to the scientific investigation of animal metabolism. He sought also to solve the practical problems of nutrition by determining the nutritive requirements of men under different conditions of living. His main experimental subject was his faithful laboratory helper Pistel, who spent his days in cleaning laboratory glassware and in tending experimental animals. Pistel was a robust man, blessed with a robust appetite, and for this reason, it is said, Voit's well-known dietary standard for a healthy man at moderate work is generously high. However, Voit's dietary recommendations are also based upon an extensive series of observations of the food habits of soldiers, prisoners, physicians, vegetarians, fat people, under-nourished people, and many others. These endeavors of Voit in Volksernährung also have been greatly extended by his pupil Rubner.

For a man of average size, healthy and performing only moderate labor, Voit recommends a daily consumption of 118 gm. of protein, 500 gm. of carbohydrate and 56 gm. of fat.

Hard workers may require up to 150 gm. of protein, 35% of which should be in the form of meat, and up to 200 gm. of fat daily. He would never recommend a consumption of more than 500 gm. of carbohydrates. With moderate activity, at least 25% of the fat consumed should be 'choice,' i.e., derived from bacon, lard or butter. With severe activity, at least 33% of the fat intake should be thus derived. Voit was decidedly opposed to vegetarianism. He freely admitted that some men could do well on less than 118 gm. of protein daily, but such men are generally light in weight, weak of constitution or physically inactive. This quota of protein for a normal man might be lowered to 108 gm. if it is largely derived from animal sources. A maintenance diet for prisoners and inmates of public institutions may contain only 85 gm. of protein, 30 gm. of fat and 300 gm. of carbohydrates.

In his practical recommendations, Voit emphasized cannily (and soundly) the importance of palatability in food. In his mind a food is first of all a palatable mixture of nutrients. His discussions of condiments (Genussmittel), including tea, coffee, alcoholic beverages, tobacco, as well as flavoring and aromatic substances, are interesting.

Condiments have a different but no less important part to fulfill in nutrition than the nutrients themselves and are as necessary as the latter for the preparation of a food. Animals and men would not usually consume a mixture of pure nutrients, but would die. As with every activity of the body, the act of taking food must be associated with a suitable sensation. The action of condiments may be compared with the lubricant for a machine or the whip for a working horse. In such a manner also the condiments function for the processes of nutrition and perform an indispensable service, although they are not able to prevent a loss of substance from the body or by their decomposition to provide us with living strength: they give us not actual strength but at most the feeling of strength by their influence on the nervous system.

It is refreshing to discover a nutrition expert who recognizes the requirements of the palate as well as those of the body.

A man's contribution to science rests not only upon the facts he establishes by experiment, but also upon his ingenuity in explaining these facts and in incorporating them into the body of knowledge of which they should be integral parts. From such explanatory theories the laws and principles of science are ultimately evolved. Voit's logical mind carried him far in explaining his experimental observations. His theories of metabolism have served a purpose in stimulating research, and as they fail to square with the newer knowledge of nutrition, they have been discarded. But it sometimes happens that the propounder of a theory considers himself its champion and is the last to admit its failure. He and his colleagues and pupils constitute a 'school of thought' dedicated to defend a theory or a group of theories against all comers. Passions are aroused, animosities are engendered and as a consequence the progress of science is impeded. The more abstruse the theory and the less amenable it is to direct experimental inquiry, the more obstinate and dogmatic its defenders become. Voit was human, intensely so, and he is no exception to this rule. His controversies with Pflüger, and especially with his old master, Liebig, are not the most inspiring phases of his career.

Voit's work and writings have contributed immensely to the evolution of our ideas concerning the fundamental nutrients and their functions in the body. According to the oldest conception, dating back to Hippocrates but persisting as late as 1833 in the writings of Beaumont, there was one 'common alimentary principle' or 'universal nutrient substance.' The recognition of 'three great staminal principles' pervades the writings of Prout (1785–1850), but no special differences in the nutritive functions of these principles were seen. The French physiologist Magendie (1785–1855) was apparently the first to demonstrate the unlike nutritive properties of the three foremost groups of organic foodstuffs. He clearly distinguished between the nitrogenous and non-nitrogenous nutrient materials. Mulder (1802–1880) coined the term 'protein' and emphasized the unique importance of the substances so grouped. Liebig (1803–1880) went still further

and exalted the importance of protein: all the energy for muscular work, glandular work and cellular work of all kinds comes only from protein. The main function of nutrition is to replace the cellular protein thus destroyed. As Verworn expressed it as late as 1899, "the life processes consist in the metabolism of the proteins;" carbohydrates and fats merely furnish animal heat.

Voit's work and writings emphasize the interchangeability of the nutrients within limits, and the sparing of one nutrient by another because of the fact that they serve like functions. According to him, the ingestion of food serves primarily to prevent the loss of protein and fat from the tissues. He believed in the conversion of protein into sugar and into fat, although, strangely, he denied the possibility of the conversion of carbohydrate into fat, believing that the experimental evidence adduced in favor of this conversion, which is considered overwhelming today, could be explained better by assuming a sparing of body fat and dietary fat by the carbohydrate of the diet.

Voit's outstanding experiments on the effect of muscular work on protein metabolism and on the respiratory exchange of man and the lower animals supplied the most telling argument against the erroneous theory of Liebig, but so imbued was he with Liebig's ideas on this point that he attempted to reconcile this evidence with the theory by proposing a mechanism by which the muscle at rest stored up electrical energy that was drawn upon during activity. He has stated emphatically: "Es muss für alle Zeiten rechtig bleiben, dass nur die organische, stickstoffhaltige Substanz krafterzeugend ist und die Bewegungsphänomene hervorbringt, und dass die Oxydation der Fette und Kohlehydrate ausschiesslich zür Wärmeproduktion, niemals aber als Energiequelle dienen könne." Only many years later was he constrained to admit the inevitable conclusion.

The fact that the heavy worker consumes more protein than the sedentary worker, and that he himself recommends a protein intake graded according to the muscular work to be

done, Voit explains not on the basis that only protein fur-
nishes energy for muscular work, but on the assumption that
the hard worker possesses a greater muscular mass than the
sedentary worker, and for the maintenance of the greater
mass of muscle he needs more dietary protein.

Voit's distinction between organized and circulating protein
explained completely the experimental facts that he himself
unearthed. With some modification it may be reconciled with
Folin's ideas of an endogenous and an exogenous protein
metabolism, and perhaps the modification required is no more
profound than that to which Folin's theory has itself been
subjected since its enunciation in 1905.

When Voit started his researches the idea was prevalent
that the oxygen supply determined the intensity of metabo-
lism, oxygen being the 'prime mover,' the 'instigator,' of
oxidation processes, combining with all substances in the
blood with which it possessed an affinity. A most noteworthy
contribution of Voit was to point out most definitely that
metabolism is not proportional to the oxygen supply, but that
the level of metabolism determines the oxygen consumption.
The horse was thus put before the cart.

Voit's experimental methods are still of basic importance.
His experimental results are still valid. His experimental
theories have either become the established principles of
nutrition or they have fallen by the wayside. Some appear
to us fantastic, but they should all be judged not by the truth
contained in them, but by the service they have performed in
harmonizing the facts established at the time of their elabora-
tion and in guiding research to the discovery of new truths.
Judged in this light the theories of Voit have served their
purposes well. Voit himself did not take his theories too
seriously, for he has said: "Die Resultate eines richtig
angestellten und richtig verwerteten Experimentes bleiben
für alle Zeiten unumstösslich, während eine Theorie im
Fortschreiten der Wissenschaft umgestossen werden kann."

H. H. MITCHELL

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THE EFFECT OF ENTERIC-COATED PANCREATIN ON FAT AND PROTEIN DIGESTION OF DEPANCREATIZED DOGS

W. A. SELLE

WITH TECHNICAL ASSISTANCE OF I. W. MOODY

Department of Physiology, University of Texas Medical School, Galveston

It is well known that exclusion of pancreatic juice from the intestine disturbs greatly the digestion of fat and protein. Thus, removal of the pancreas or occlusion of its ducts in animals leads to the loss of large amounts of fat and nitrogen in the feces, to a rapid and marked loss of body weight, and ultimately to death unless special care and diets are given. The addition of fresh or dried pancreas to the diet checks the excessive waste of food and body tissue and permits the animal to live indefinitely.

Although pancreatin has been used clinically for many years in cases of enzymatic deficiencies of the pancreas, results with the usual preparations have not been very successful (Bastedo, '25). In vitro experiments have shown that many enzymes are rapidly inactivated or destroyed by other enzymes and by an excess of hydrogen or hydroxyl ions. That trypsin may be digested by pepsin has been reported by Long and Johnson ('13), by Long and Hull ('16), by Northrop and Kunitz ('32), by Kleiner and Tauber ('34), and others. Long and Johnson ('13) showed that while trypsin is not destroyed or weakened by the addition of 0.2% HCl, it is greatly weakened by the presence of the acid and pepsin. That pancreatic lipase and amylopsin are destroyed in vitro by acid was reported by Lisbonne ('33). Schulman and

Rideal ('33) found that pancreatic solutions are digested by pepsin at pH 2.0. Kleiner and Tauber ('34) subjected pepsin and trypsin to reciprocal digestion and demonstrated that at pH 2.0 pepsin completely digests trypsin, leaving the pepsin unchanged. At this pH trypsin is inactive but not destroyed. If, however, a mixture of the two enzymes is kept at pH 5.5, which does not permit peptic activity, the pepsin is digested by trypsin.

Based on the assumption that the enzymes of pancreatin are inactivated in vivo by gastric juice, Sansum ('26, '32) has advocated the use of enteric-coated pancreatin granules and tablets in the treatment of certain forms of indigestion. The coating of these preparations presumably protects the contents from the destructive effect of gastric digestion, yet allows for immediate dissolution of the active enzymes in the intestine.

The present absorption studies were undertaken to determine the efficacy of enteric-coated pancreatin preparations in animals in which pancreatic juice is entirely excluded from the intestine by removal of the pancreas. Depancreatized dogs develop a greatly improved gastric digestion, and an increased gastric and intestinal acidity (Yesko, '28, and Fauley and Ivy, '29). These animals, therefore, make excellent experimental subjects for testing the hypothesis that pancreatic enzymes are inactivated by gastric juice and for testing the efficacy of enteric-coated preparations of pancreatin.

METHODS

Totally depancreatized dogs, weighing between 9 and 12 kg. and sustained by the administration of insulin, were fed 50 to 70 gm. of fresh beef per kilogram of body weight per day. In order to avoid marked fluctuations in absorption known to occur in depancreatized dogs and to obviate digestive disturbances, such as vomiting and diarrhea, only lean meat was fed. This highly palatable food was usually administered in three equal feedings, evenly spaced during the day. In later experiments only two feedings, at 8 A.M. and 8 P.M., were

given. The meat was thoroughly minced and mixed by grinding five or six times and was prepared in 80 pound lots and kept in tightly sealed jars at a temperature of 33°F. until used. The fat content of seven lots of meat prepared varied between 2.5 and 8.5%, the protein content between 18 and 21%.

Two preparations of pancreatin were used, small granules (0.5 to 1 mm. in diameter) and 5 grain tablets. Each was made up in two forms; one was coated, the other was not and thus served as control material. The pancreatin granules (both plain and enteric-coated) were preferred to the tablets since the former mix more thoroughly with the gastric contents and on passing into the intestine afford several hundred foci from which digestion takes place, instead of one, as in case of the tablet. The enteric-coating of the preparations is reported by the manufacturers to be composed of a resinous substance soluble in alcohol and alkalies, its solubility being tested in a 2% sodium bicarbonate solution having a pH of 8.4. Sansum ('32) previously showed by x-ray technic that this coating remains intact as long as the pellets stay in the stomach, but that it soon dissolves after reaching the small bowel.

Although the pancreatin employed in the present studies was obtained from two sources, the coated and uncoated (control) preparations for any one experiment were taken from the same manufactured lot and were consequently of the same strength. Standardization tests made on fresh material showed the proteolytic strength of the various preparations to be between 250 and 325% according to the casein assays of the tenth revision of the U.S.P. Although a satisfactory method of assay for lipase has never been devised, attempts were made to determine the relative effectiveness of the coated and uncoated granules on olive oil at varying H-ion concentrations.[1] The titration figures of five assays carried out

[1] Steudel ('33) has stressed the difficulties of assaying lipase and agrees with Willstätter that the only way to estimate the content of this enzyme is to construct a curve of weight and saponifying power for any given preparation, and to utilize its form as a basis of assay.

according to the method of Cherry and Crandall ('32) show such marked variations from experiment to experiment that average values have little meaning. For any one experiment, however, the figures indicate, as shown by a typical experiment summarized below, that for a pH range of 5.6 to 8.4 the two preparations of pancreatin have the same lipolytic strength.

Assay of lipase. Two hundred milligrams of pancreatin and an emulsion containing 7 cc. pure olive oil, 5% gum acacia and a buffered solution, were incubated at 40°C. for 18 hours. The titration figures for the fatty acids liberated are expressed in cubic centimeters 0.2 N NaOH.

	pH 5.6	7.0	7.8	8.4
200 mg. plain granules	18.0	28.3	40.7	35.9
200 mg. coated granules	17.1	27.6	41.3	36.3

In several preliminary experiments increasing amounts of pancreatin were given for the purpose of determining the amount required to complete fat digestion. The addition of 6 or more gm. of pancreatin per 100 gm. of meat frequently produced a severe digestive upset with loss of appetite, marked diarrhea and the passage of large masses of mucus in the stools. The amount of pancreatin given in most experiments reported here was 3 gm. for each 100 gm. of food.

Each experiment was divided into three parts or absorption periods, one in which food alone was given, another in which 3 gm. of uncoated pancreatin (granules or tablets) was given with each 100 gm. of meat, and a third in which an equivalent amount of the enteric-coated pancreatin was given. The order of these parts was changed from time to time. In some experiments the pancreatin was well mixed with the food before feeding, in others it was given after feeding. Each absorption period lasted for six to ten feedings and was demarked by inclusion of charcoal or carmine in the diet.

The animals were trained to defecate on a glazed tile floor so that the feces might be collected easily, quantitatively and immediately following elimination. This procedure had the further advantage of making possible the determination of the elimination time of the feces as the animals were taken

from their individual cages every 3 or 4 hours during the day to permit defecation. Although the stools were usually firm, diarrhea occurred occasionally and the entire absorption period was repeated. Following their collection at the time of elimination, the feces were weighed and preserved with sufficient 20% formalin to give a final formalin concentration to the fecal material of 5%, which is well in excess of the amount necessary to inhibit enzymatic and bacterial action. The pooled samples were kept in air-tight containers in the refrigerator until analyzed.

Since drying of feces results in the loss of ammonia as well as certain fatty acids (Kleiber, Caldwell and Johnson, '36) analyses of fat and nitrogen were usually made on moist feces. In later experiments in which fat alone was determined, the feces were preserved in 95% alcohol and dried over a steam bath. Nitrogen was determined by the usual Kjeldahl method, no correction being made for the nitrogen of the ferment itself. The total ether soluble fats were estimated by the method of Saxon. Although the unsaponifiable fats were estimated in most of the experiments, their value in the present study is questioned and special consideration will not be given them here. All determinations were made in triplicate. The nitrogen and fat of the food were determined by the same methods used in the analysis of the feces.

RESULTS

Lack of space does not permit of the giving of complete results for individual animals. Summaries of fifteen experiments on four dogs, the feces of which were analyzed wet, and of thirteen experiments on six dogs, the feces of which were analyzed dry, are presented in tables 1 and 2, respectively. The figures show changes in the quantity of feces voided and the amount of nitrogen and fat recovered from the diet of lean meat with and without digestive aid (pancreatin).

Weight of feces. The decreased weight of the feces after inclusion of pancreatin in the diet is an outstanding feature of all experiments. Despite the fact that the stools vary

greatly in water content, thus rendering any comparison of weight on wet feces untrustworthy, their reduction in weight with pancreatin was so marked (25 to 60%) in the fifteen experiments in which the feces were analyzed moist (table 1) that mention is made. There was, however, no consistent and significant difference between the weights of wet feces recovered during administration of the coated pancreatin and those recovered during the administration of the uncoated. A more accurate comparison based on the thirteen experiments in which the feces were dried, shows the average decrease in weight after pancreatin was approximately 55%; in several experiments the decrease was well over 60%. In three of nine experiments permitting comparison, the feces collected during inclusion of coated pancreatin weighed slightly more than those collected during administration of the uncoated, in three experiments the reverse was true, and in three the weights were the same. Thus, pancreatin reduces greatly the quantity of feces, but there is no apparent difference between the action of coated and uncoated preparations in this respect.

Elimination time. Another marked change produced by the inclusion of pancreatin in the diet was the definite increase in elimination time, figures for which are not included in the tables. Whereas the elimination time for these dogs when not receiving pancreatin was 12 to 18 hours, during pancreatin feeding it was increased to between 20 and 30 hours. Whether this increased elimination time is due to the reduction of intestinal contents resulting from improved protein digestion, whether the pancreatic enzymes exert a specific inhibitory action on the motility of the alimentary tract, or whether other factors are involved, is not evident from these experiments. Fauley and Ivy ('29) noted that the emptying time of the stomach is decreased more by total pancreatectomy than by ligation of the pancreatic ducts and concluded that hunger is the chief cause of the stomach opening sooner in the absence of pancreatic juice, acidity of the duodenum being less important.

TABLE 1

Efficacy of pancreatin on fat and protein digestion of depancreatized dogs

SERIES	DOG NO.	Number of feedings	Total meat fed	Total fat fed	Total 'N' fed	Pancreatin added per 100 gm. food	Weight of moist feces [1]			Ratio of fecal fat to food fat			Ratio of fecal 'N' to food 'N'		
							Without pancreatin	With plain granules	With coated granules	Without pancreatin	With plain granules	With coated granules	Without pancreatin	With plain granules	With coated granules
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	%	%	%	%	%	%
1A	1	10	2000	50.0	59.24	3 granules	183	145	117	18.7	32.6	21.8	15.2	11.6	10.8
	2	8	1600	40.0	55.38	3 granules	174	106	100	23.8	28.1	26.2	20.0	12.0	9.4
	3	8	1600	40.0	55.38	3 granules	197	115	107	18.2	29.2	31.8	18.4	12.0	12.4
	4	8	1600	40.0	55.38	3 granules	224	178	176	22.8	33.6	37.2	32.9	13.4	15.3
2A	1	6	1800	57.6	54.33	3 granules	392	190	209	19.5	20.8	21.8	36.0	8.5	10.5
	2	6	1200	38.4	36.22	3 granules	258	192	191	20.4	40.0	35.0	32.4	20.8	21.6
	3	6	1800	57.6	54.33	3 granules	439	162	193	28.6	31.0	22.4	23.3	8.8	8.4
3A	1	9	1800	153.0	57.75	3 granules	540	459	420	27.0	39.0	32.9	27.5	12.2	13.4
	2	8	1600	136.0	51.20	3 granules	528	379	357	29.7	45.8	37.7	31.0	13.8	14.8
	3	8	1600	136.0	51.20	3 granules	535	380	362	25.6	45.2	36.0	29.9	16.0	13.5
	4	8	1600	136.0	51.20	3 granules	459	302	308	43.3	48.7	52.6	27.6	18.5	14.8
4A	1	6	1800	78.3	56.76	3 tablets	566	380	266	31.6	33.8	25.6	27.2	15.1	10.4
	4	6	1800	78.3	56.76	3 tablets	455	228	243	20.4	26.8	23.5	25.0	10.3	9.0
5A	1	7	2100	91.3	67.60	3 tablets	543	332	374	22.0	28.4	31.1	29.4	10.6	15.3
	4	8	1600	69.6	51.39	3 tablets	328	246	223	18.3	22.4	31.4	21.2	11.8	10.8

[1] Nitrogen and fat were determined on wet feces preserved with formaldehyde.

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Nitrogen absorption. Following the addition of pancreatin to the diet, fecal nitrogen was diminished by 35 to 65%, the average reduction being approximately 52%. Thus, protein digestion was markedly and uniformly improved by the pancreatin. That there was no significant difference, however, in the effectiveness of the two pancreatin preparations on protein digestion, is indicated by the fact that while the coated reduced fecal nitrogen more (by 2% or more) than did the uncoated in four experiments, the reverse was true in three experiments and the values were approximately the same for the remaining eight.

Fat absorption. The figures indicate that fat digestion is not benefited by the addition of either the coated or uncoated pancreatin, and, as a matter of fact, its addition in the amounts given suggests that inhibition of fat digestion occurred in some experiments. Thus, in fifteen experiments in which the feces were analyzed wet, the average recovery of fat without pancreatin was 24.6% of the ingested fat, as compared with 31.1 and 33.7% for the coated and uncoated pancreatin, respectively. Without pancreatin the fecal fat was definitely less (by 4% or more) in nine experiments, more in two experiments and approximately the same as with pancreatin in the remaining four. Comparing the figures for the coated and uncoated pancreatin, we find the recovered fat was less (by 4% or more) for coated pancreatin in seven experiments, but more in three experiments and the same as the uncoated in five.

While values for experiments in which the feces were dried (table 2) are considerably lower than those obtained on the wet feces, the same general results are noted, namely, that fat elimination is not checked by pancreatin, and that the plain and coated pancreatin give approximately the same results. Three experiments of this series show less fecal fat without pancreatin than with, two show more, and eight have values which are the same as those for pancreatin. Averaging the figures, we find that 10.49% of the ingested fat was recovered without pancreatin, 11.02% with coated and 11.42 with the uncoated.

TABLE 2

Efficacy of pancreatin on fat digestion of depancreatized dogs

SERIES	DOG NO.	DIET					FECES					
		Number of feedings	Total meat fed	Fat content	Total fat fed	Pancreatin fed per 100 gm. food	Dry weight			Ratio fecal fat to food fat		
							Without pancreatin	With coated granules	With plain granules	Without pancreatin	With coated granules	With plain granules
			gm.	%	gm.		gm.	gm.	gm. N	%	%	%
1B	5	7	2100	5.6	117.6	3 granules	176.8	76.3	77.0	15.86	21.40	18.70
	6	7	2100	5.6	117.6	3 granules	106.6	60.2	...	12.75	11.48	....
2B	5	8	2400	4.2	100.8	3 granules	215.0	71.0	60.5	13.66	15.31	17.61
	7	8	2400	4.2	100.8	3 granules	195.0	78.6	...	25.47	26.92	....
3B	5	6	2400	5.1	122.4	2½ granules	222.5	67.0	75.3	15.40	12.90	13.75
	7	6	2400	5.1	122.4	2½ granules	148.5	77.7	82.1	8.34	12.36	12.67
	8	6	2400	5.1	122.4	2½ granules	92.0	76.8	72.2	9.54	11.08	10.53
4B	5	6	2400	5.1	122.4	2½ granules	145.0	71.0	65.0	8.88	8.04	7.37
	8	6	2400	5.1	122.4	2½ granules	76.0	51.0	54.0	6.02	5.70	6.22
5B	5	7	2100	5.54	116.3	3 tablets	123.0	62.0	46.0	10.39	8.10	9.78
	8	7	2100	5.54	116.3	3 tablets	58.0	40.5	45.0	7.88	7.83	9.78
	10	7	2100	5.54	116.3	3 tablets	66.0	44.0	41.0	8.96	7.49	8.02
	12	7	2100	5.54	116.3	3 tablets	270.0	...	181.0	47.60	....	44.50

Additional observations. In several preliminary experiments increasing amounts of pancreatin were used to determine the amount necessary to complete the digestion of fat. It was soon learned that regardless of the amount given, fat was not completely digested; in fact, large amounts seemed less effective than small. Protein digestion, however, was improved with larger amounts. Thus, in two experiments on two dogs, the N recovered from the feces was 27.7 and 27.4% of the ingested N. With 3 gm. of pancreatin per 100 gm. meat the percentage of N recovered dropped to 16.6 and 14.2, and with 6 gm. it was 12.8 and 10.4. In a third experiment, in which the increase in pancreatin was in 2 gm. amounts, the N recovered without digestive aid was 29.4% of the ingested N, with 2 gm. pancreatin it was 15.8, with 4 gm. 10.3, and with 6 gm. 9.5. Similar results were reported by Nasset, Pierce and Murlin ('31). That the improvement is not more striking with the larger amounts is probably due to the fact that, although the degree of acceleration of a hydrolytic reaction is proportional to the concentration of the enzyme present, the final amount of products formed is independent of the amount of catalyst. Time and the initial concentration of the substrate are also important factors controlling the amount of food undergoing transformation in the alimentary tract.

Mention should be made of several observations on the passage from the intestine of intact enteric-coated tablets. On one occasion the stool of dog no. 3 and on three occasions the stools of dog no. 8 contained whole tablets. As the passage of the tablets occurred without any accompanying intestinal disturbance, evidenced by the fact that the animals ate well at the time and were free of diarrhea, the occasional failure of the resinous coating to disintegrate is of interest. The manufacturers of the tablets have also noted a variation in the solubility of the enteric coating in different individuals. Their observations with the fluoroscope showed that in most patients the tablet passes through the stomach and disintegrates readily in the small intestine, although in some patients

the same tablet disintegrates in the stomach, and in still others it is carried through the intestinal tract intact (Bibbons, '34).

GENERAL DISCUSSION

That fat digestion is not greatly impaired in dogs deprived of pancreatic juice and given moderate amounts of fat was also reported by Handelsman, Golden and Pratt ('34) on duct-ligated animals. Since in the absence of pancreatic juice as much as 50 gm. of fat may be absorbed daily by a 10 kg. dog, it is apparent that the lipases of gastric and intestinal juices play an important role in the digestion of fat.

An explanation of the failure of pancreatin to improve fat digestion is wanting. That fat absorption may even be made worse by its inclusion in the diet is indicated by the fact that in many experiments more fat was recovered from the feces with pancreatin than without. It is possible that the proteolytic enzyme of the pancreatin inactivated to some degree the gastric and intestinal lipases. Falk ('33) has found that trypsin greatly decreases the effect of lipase on various substrates. Insufficient knowledge of the lipases, however, does not justify further theorizing along this line. Experiments in which only pancreatic lipase is given are now in progress and will be reported later.

That the proteolytic enzyme of the coated pancreatin is no more effective than that of the uncoated is probably explained on the grounds that the presence of food in the stomach offers protection to the enzyme from the gastric juice, and in this respect the in vitro experiments mentioned above differ fundamentally from these. Macleod ('20) states:

If pepsin is present together with trypsin in a distinctly acid solution, the pepsin seems to destroy the trypsin, unless the mixture contains a considerable quantity of protein, when the tryptic activity may persist even for several hours. A practical conclusion which we may draw from these results is the effect that preparations of trypsin—the so-called, *pancreatin,* for example,—if given with the food, may pass in an active condition into the duodenum, where, in the more favorable environment created by the neutralization of the excess of acid, it will develop its proteolytic power. The therapeutic administration of pancreatin is, therefore, justified.

· Inactivation of enzymes by changes in the H-ion concentration and by other enzymes is probably not a simple assault on the enzyme itself, as various authors have pointed out. Thus, Willstätter ('23) found that lipase extracted from gastric mucosa exhibited optimum activity at pH 5.5 to 6.3 and complete inactivation at pH 8.6. After repeated purification with kaolin, the optimum pH was found to be near 8.0, or identical with pancreatic lipase. The enzyme, as secreted in the stomach, is presumably associated with a substance which inhibits its action in a more alkaline medium. According to Willstätter and associates ('22–'24), enzymes are associated with carriers which are chiefly protein in nature, and which have nothing to do with the catalytic effect of the enzyme per se. These workers attribute the inactivation of enzymes by proteases to digestion of the protein carriers, not the enzymes themselves. In pure form many enzymes contain little nitrogen, or else are free of protein (Tauber, '35). Kleiner and Tauber ('34) have recently obtained a trypsin preparation which does not contain protein of the ordinary type, as indicated by the biuret, Millon and heat coagulation tests. Northrop and Kunitz ('34) have also described a highly active crystalline preparation of trypsin, which suffers no loss of activity and no denaturation when a solution is heated in acid to boiling.

In the test tube and under ideal conditions pepsin may inactivate trypsin by digesting its carrier, or by assaulting the free enzyme itself. In the stomach containing food, however, peptic digestion is certainly not carried sufficiently far to bring about irreversible inactivity of trypsin. Hence, trypsin of uncoated pancreatin is as effective as that of the coated, regardless of whether the pancreatin is mixed with the food before ingestion or after. The addition of an enteric coating to pancreatin, therefore, seems unnecessary. In some instances the coating may be definitely disadvantageous, as it occasionally fails to disintegrate in the small intestine and thus prevents liberation of the enzymes.

SUMMARY

The effectiveness of several commercial preparations of plain and coated pancreatin on the digestion of ten totally depancreatized dogs, sustained with insulin, was studied. The addition of 3 gm. of pancreatin per 100 gm. of fresh ground meat 1) reduced the quantity of feces by 30 to 60%, 2) increased the elimination time to approximately normal values, 3) reduced the nitrogen loss of the feces by 30 to 60%, but 4) failed to check the loss of fat in the feces. Larger amounts of pancreatin were more effective than small in the digestion of protein, but were no more effective, possibly less effective, than small in the digestion of fat. There appears to be no uniform significant difference between the action of coated and uncoated pancreatin on digestion of fat and protein.

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ON THE EFFECTIVENESS OF ORALLY ADMINISTERED DIASTASE IN ACHYLIA PANCREATICA (DOG)

J. M. BEAZELL, C. R. SCHMIDT AND A. C. IVY

Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago

Although there are a few reports in the literature which show that the oral administration of raw pancreas or pancreatin reduces the fat and nitrogen lost in the feces in pancreatic achylia (Ivy, '35), we have been unable to find a worthy report showing that orally administered diastase is effective in reducing the loss of starch. This work was undertaken to obtain controlled evidence on the question, using diastases which are inactivated at a relatively low and high pH.

METHODS

Chemical method for estimating the starch and reducing sugar content of the feces. The starch and reducing sugar content of the feces was determined by a method devised by us. The method is based on the following principles: The feces are dried with alcohol and ether, thoroughly mixed, and an aliquot is suspended in a buffer solution and boiled to render the starch soluble. The mixture is then digested with Takadiastase until all of the starch has been changed to reducing sugars. The mixture is then centrifuged, sugar determinations are made on the supernatant fluid, and the starch content of the feces is calculated.

The feces are collected as soon as possible after passage and macerated with approximately 5 volumes of alcohol.

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When convenient the mixture is filtered with suction and washed with additional alcohol. The residue on the paper is washed with ether, placed in a beaker and set aside to dry. This method of drying the feces was used because it is quicker and removes certain of the non-sugar reducing substances. The dried feces are then weighed and two 1-gm. samples are taken for the starch determination. Each sample is placed in a 250-cc. flask containing 90 cc. of pH 4.5 buffer solution, and refluxed for 10 minutes. This insures complete solution of any starch present. The fecal suspension is then cooled. Ten cubic centimeters of an active 2% solution of Takadiastase[1] is added to one of the samples. To the other is added 10 cc. of a 2% solution of Takadiastase which has been previously boiled. The latter sample serves as a control to estimate pre-formed and non-sugar reducing substances. These samples are then incubated at 37° for 2 hours.

At the end of the incubation period the samples are centrifuged and the supernatant fluid analyzed for reducing substances. Although any reducing sugar method may be used for this determination, we have used the hypoiodate titration method as modified by Schmidt, Greengard and Ivy ('34). In this method 5 cc. of the supernatant fluid is added to 10 cc. of N/10 iodine. This mixture is then alkalinized by slowly adding 50 cc. of N/8 NaOH with constant shaking. It is set aside for 15 minutes then acidified with 2 cc. of 10% sulphuric acid and the excess iodine titrated with N/20 sodium thiosulphate.

The reducing substance in the two samples, expressed as maltose, is then calculated according to the following formula:

$$\frac{(10 - \frac{T}{2}) \times 17.15 \times 100}{5} = \text{mg. reducing substance,}$$

as maltose, in 1 gm. sample of feces.
T = sodium thiosulphate titration value

The reducing substance value for the control is then subtracted from the reducing substance value of the test determination, leaving the reducing substances, expressed as

[1] Commercial Takadiatase contains a diluent. The product used by us was obtained in pure form from Parke, Davis & Co.

maltose, that have been produced by the digestion of starch. After repeated determinations we have found that 1 gm. of starch treated by this method will yield approximately 1.4 gm. of reducing substance expressed as maltose. The quantity of starch in the 1-gm. sample of feces analyzed is calculated by dividing the maltose value obtained above by 1.4.

The accuracy of the method was tested by analyzing samples of feces from normal dogs and men to which known quantities of starch had been added. The result of one such series of determinations is given in table 1. The maximum error of the

TABLE 1

SAMPLE NUMBER	CALCULATED STARCH CONTENT	DETERMINED STARCH CONTENT	RECOVERED
	%	%	%
1	30	27.4	91.3
2	30	30.4	101.0
3	30	30.0	100.0
4	30	31.8	106.0
5	30	28.7	95.6
6	30	30.5	101.5
7	30	29.4	98.0
8	40	41.1	102.7
9	40	36.5	91.2
10	40	35.3	88.3
		Average	97.6

[1] Numerous series of such tests were made and the results in this table show the maximum differences.

method in our hands is $\pm 11\%$, which is more accurate than other quantitative methods we attempted to employ.

The conversion factor, 1.4, was determined by digesting a sample of cornstarch to reducing sugar according to the method. In other words 1 gm. of the cornstarch we used will yield 1.4 gm. of reducing sugar expressed as maltose. This does not mean that all forms of starch will have this same conversion value. It should be remembered, therefore, that the values obtained by this method are in terms of cornstarch and may not represent the true quantity of other types of starch. The method possesses one other inherent limitation. In addition to digesting the starch to sugar, the Takadiastase

digests small quantities of some other substance, probably protein, to reducing substances. Thus, we have consistently obtained values of from 0.5 to 2.0% 'starch' in samples of feces that have been proven by microscopic examination and the iodine test to be free of starch. Takadiastase is used in the method in spite of this limitation because after repeated tests using different enzyme preparations it proved to be most satisfactory. Since the maximum error of the method is only ±11%, we consider our experimental data on the dogs to be accurate, and greatly within the biological variations.

Physiologic method. Six dogs made totally deficient in external pancreatic secretion by separation of the pancreas from the duodenum were employed. The dogs were then given 1 kg. daily of a diet containing 62% starch (dry weight). The diet consisted of farina cereal, 12%; cod liver oil, 1.6%; lard 1.6%; canned salmon, 9.0% (wet weight); water 75.8%; salt to flavor. The mixture was cooked and prepared in large batches and kept in the ice box. The feces were collected fresh and the 24-hour excretion analyzed for starch. After the dogs were placed on the diet for several days, a control period was started and then the various diastase preparations were given. The feces passed for the first 3 days of any test period were discarded. Four control periods were conducted on most of the dogs.

Potency of enzyme preparations used. A number of diastase-containing enzyme preparations were assayed for potency; some were found inactive or practically so. Only those of strong potency and easily obtained were chosen for our experiments. The potency of the different preparations used are shown in table 2.

Takadiastase (Parke, Davis & Co.) was selected because it is inactivated at a low pH (pH 2.4) and because it is used clinically. Desiccated malt extract was chosen because it is inactivated at a similarly low pH (pH 2.5) and is a relatively inexpensive source. A commercial food preparation (Ovaltine) was chosen for the same reason, since we found it to contain appreciable diastatic power. Pancreatin was tested

because it is used clinically and is inactivated at a higher pH than the plant diastases, and for this reason differences might be found. Enteric coated pancreatin tablets were used to ascertain if the coating increased effectiveness.

The various enzyme preparations were administered in the doses shown in table 3. Half of the dose was mixed with the test meal immediately before feeding and the remainder was given in or with water after the meal had been ingested. The total daily dosage of each preparation expressed in terms of saccharogenic and liquefying power is given in table 2. This method of administration was used because of results obtained in a previous study (Ivy, Schmidt and Beazell, '36).

TABLE 2

PREPARATION	WILLSTATTER UNITS PER GRAM	TOTAL DAILY DOSE IN WILLSTATTER UNITS	GRAMS STARCH HYDROLYZED IN 15 MINUTES AT 37° C. PER GRAM OF PREPARATION	GRAMS STARCH HYDROLYZABLE TO MALTOSE BY TOTAL DAILY DOSE (IN 15 MINUTES)	STARCH LIQUEFYING UNITS (DAVIDSON) PER GRAM	TOTAL DAILY DOSE IN LIQUEFYING UNITS
Pancreatin						
Enteric tablets	7.20	180.0	35	875		
Powdered	6.20	155.0	32	800	55,000	1,375,000
Dry malt extract	1.60	40.0	9	225	3,060	76,500
Takadiastase	0.96	24.0	5.5	137	11,260	281,500
Commercial food preparation	0.56	28.0	3.5	175	2,200	110,000

Greater effectiveness might have been obtained, if further enzyme had been administered with milk, which buffers acid, at 2 and 3 hours after the meal. We did not do this because it would have meant treating the dogs during the control period with milk at those hours.

RESULTS

Prior to the separation of the pancreas from the duodenum the dogs passed no starch in their feces while receiving the high starch diet. After the separation of the pancreas, the dog's feces contained from 18 to 39% starch as may be noted in the columns headed 'first series,' table 3. The dogs divide

TABLE 3

	FIRST SERIES OF TESTS				SECOND SERIES		
	DOG NO.	Days on test	Average starch per 100 gm. feces per day	Per cent decrease in fecal starch	DOG NO.	Days on test	Average starch per 100 gm. feces per day	Per cent decrease in fecal starch
Starch diet	1	4	33.4		1	16[1]	33.9	
alone					2	16	19.1	
Control	3	4	33.4		3	16	29.3	
	4	4	18.0		4	16	19.0	
	5				5	16	19.6	
	6	3	39.2		6			
Average			25.6				24.2	
Starch diet	1	4	13.7	59.6	1	3	13.9	59.0
with Takadia-	3	4	14.6	56.3				
stase 25 gm.	4	4	12.8	29.0	4	3	11.5	40.0
	6	4	14.9	61.8				
Average			14.0	51.6			12.7	49.5(?)
Starch diet	1	4	16.1	51.8				
with malt ex-	3	4	16.7	50.0				
tract 25 gm.	4	4	14.3	20.5				
	6	4	16.5	57.9				
Average			15.9	45.0				
Starch diet	1	4	20.2	39.5	1	14[2]	17.4	48.7
with commer-	2				2	14	7.6	60.0
cial food	3	4	19.9	40.5	3	14	17.1	41.7
preparation	4	4	15.5	14.0	4	14	17.7	7.0
50 gm.	5				5	14	10.5	46.5
	6	4	20.2	48.5	6			
Average			18.9	35.6			14.0	40.7
Starch diet	1	4	25.2	24.5	1	5	26.4	22.0
with Pan-	3	4	16.7	50.0				
creatin 25 gm.	4	4	14.8	18.0	4	5	12.5	34.0
	6	4	12.5	68.1				
Average			14.7	40.1			19.4	28.0(?)
Starch diet	1	4	14.6	56.5				
with Enteric	3	4	14.4	57.0				
Pancreatin	4	4	13.7	23.7				
tablets 25 gm.	6	4	14.3	63.5				
Average			14.0	50.1				

[1] Average of one 8-day and two 4-day control periods.
[2] Average of two 7-day test periods.

themselves into two groups in regard to a high (dogs 1, 3, 6) and low (dogs 2, 4, 5) loss of starch. It is to be noted that each dog from one control period to another was remarkably constant in regard to the percentage of starch lost in the feces. Table 4 shows the results of a typical test on dog 1, and is given to show the variation from day to day. To conserve space, three of the control periods are averaged in the column headed 'second series' in table 3. The second series of tests with diastase (Takadiastase, pancreatin and commercial food preparation) yielded results that as a whole check as well as could be expected, except in the case of dog 4.

TABLE 4

Typical series of determinations showing daily variation in starch excretion. Dog no. 1

STARCH DIET ALONE		STARCH DIET PLUS COMMERCIAL FOOD PREPARATION	
Day of diet	Starch per 100 gm. feces	Day of diet	Starch per 100 gm. feces
1	34.2	1	14.3
2	19.4	2	18.1
3	29.0	3	18.3
4	33.7	4	33.3
5	51.0	5	19.5
6	42.0	6	19.4
7	28.0	7	19.0
Average	33.9	Average	20.1

The results in table 3 are expressed on a percentage basis because, since the stools are frequently mushy, it was technically impossible to collect all of the feces each day. However, calculations were made of the percentage of ingested starch actually lost, and the results, as might be expected, were roughly proportionate to the percentage changes. Only the percentage figures are given in the tables because we are certain of their accuracy within the limits of our method. The effect of the addition of enzymes is very definite. Averaging the results of series one and two on Takadiastase the percentage reduction in fecal starch is 50; the percentage reduction with malt extract is 45 and with the commercial food

preparation is 38.5. The average percentage reduction with pancreatin powder is 36.1 and with the enteric coated pancreatin tablets 50.1.

DISCUSSION

The oral effectiveness of diastase in reducing the starch loss in the feces of dogs with pancreatic achylia is quite obvious from the results. This might be expected since we (Ivy, Schmidt and Beazell, '36) have shown that when malt amylase, or even ptyalin, is administered with a meal a considerable portion of it passes into the intestine before the pH of the gastric contents is lowered to the point of inactivation of the enzyme.

Although the averages indicate that Takadiastase may be slightly superior to malt diastase, in view of the fact that fewer saccharogenic units of the former were administered, we doubt that our data permit such a deduction. Further, the greater liquefying power of Takadiastase as compared with malt diastase must be considered. However, we do believe that malt diastase (45%) is more effective than pancreatin powder (36.1%) on the basis of the difference (8.9%) in the averages and the large differences in the units of enzyme (800 units of pancreatin and 225 of malt extract) administered. This is not surprising in view of the fact that pancreatic diastase is inactivated at a much higher pH relatively than malt diastase. The data show very clearly that the enteric coating increases the effectiveness of pancreatin.

The differences between the dogs, of course, is to be expected, because it is well known that some dogs withstand pancreatic achylia much better than others.

Attention should be directed to the relatively large doses we employed to obtain the above results and the fact that in no instance was the starch completely eliminated from the feces. On a lower starch diet and in dogs whose gastric acidity does not rise rapidly post-cibum, it is probable that smaller doses would completely eliminate starch from the

feces. Also, amylorrhoea would probably not be as marked in the human in pancreatic achylia as in the dog because the dog produces no ptyalin.

SUMMARY AND CONCLUSIONS

Absolute pancreatic achylia was produced by separating the pancreas from the duodenum in six dogs, which were fed a high starch (62%) diet. Prior to the production of the achylia the dogs did not show amylorrhoea while on the diet. After the production of the achylia the starch in the feces varied from 18 to 39% in the different dogs. The administration of diastatic enzymes in relatively large amounts very definitely reduced the starch loss in each dog, proving the effectiveness of oral diastatic therapy in pancreatic deficiency. The vegetable diastases were more efficient than pancreatic amylase (pancreatin) which is most probably due to the latter being more readily inactivated by the gastric acidity than the former. Enteric coating of the pancreatic amylase (pancreatin) rendered it as effective as the vegetable diastases; but the enteric coated pancreatin was not as effective as the vegetable diastases on the basis of the enzymic units administered.

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THE BILATERAL SYMMETRY OF SKIN TEMPERATURE

H. FREEMAN, F. E. LINDER AND R. F. NICKERSON

Research Service of Worcester State Hospital and Memorial Foundation for Neuro-endocrine Research, Worcester, Massachusetts

Previous investigations of the physiological state of the schizophrenic patient have shown an abnormal variability of various processes and, in certain respects, a displacement of mean functional levels (Hoskins and Jellinek, '33). In Cannon's terminology, the schizophrenic patient seems to show abnormal homeostasis. Such adaptative inefficiency lends itself readily to quantitative analysis from the viewpoint of the effects of varying temperature, humidity and air velocity upon the heat regulating mechanisms.

A dysfunction of heat regulation in this psychosis is suggested by several studies. Gottlieb and Linder ('35) have found that on exposure to changes in the environmental temperature from 73°F. to 93°F. patients exhibit significantly greater increases than do those of normal individuals. Carmichael and Linder ('34) have noted that there is a higher correlation between oral and rectal temperatures in schizophrenic subjects than in normal controls, thereby implying in the former group a lessened adaptability to environmental or internal variations. Hoskins and Walsh ('32) have reported a decreased oxygen consumption rate, i.e., a diminished heat production in this psychosis. Finkelman and Stephens ('36) have observed in a study of the response to cold in schizophrenic and normal subjects that in the former group the heat

production was lower, that it was not maintained as long, and that there was no reactive hyperemia.

The experiments to be described in this and subsequent papers have been studies of the skin temperatures, body temperatures and oxygen consumption rates of normal and schizophrenic individuals under varying environmental conditions. Many temperature studies of normal individuals have been reported. Since few have been performed with a sufficient number of subjects or under adequately controlled environmental conditions to give statistically reliable values, it has been necessary to establish our own normal standards.

The environmental control was secured by the construction of a psychrometric laboratory provided through the generosity of the Rockefeller Foundation. In this laboratory the equipment was designed to produce on any day in the year dry bulb temperatures varying from 10°C. to 50°C. with relative humidities varying independently from 20% to 90% throughout the temperature range. The air velocity could be held constant or could be varied through a wide range.

For the study of temperatures copper-constantin thermocouples were used, one for skin, two for oral and two for rectal determinations. The various thermocouples were connected by a multiple switch so that all could be read in rapid succession through the medium of a galvonometer calibrated to be read in temperatures, directly. The oxygen consumption rate was determined by means of a Benedict-Roth metabolism instrument. The air velocity was measured on several occasions each day by means of a kata-thermometer (Angus, Hill and Soper, '30). In order to ensure as nearly complete access as possible of all environmental factors to the entire surface of the body, the beds were constructed of coarsely woven cane with wide interstices. No mattresses, blankets or sheets were used. For the convenience of the subjects sponge rubber mats were placed under the head and heels.

Forty subjects were used for the experiments, twenty being healthy men and twenty men suffering from schizophrenia, but otherwise without disease. The control and psychotic subjects were approximately matched for height and weight.

They lived in adjacent rooms, took approximately the same amount of exercise and ate the same type of food. Thus for each group the extra-experimental conditions were quite comparable.

Each morning at 9.00 A.M. a patient and his matched normal control were conducted from their beds to the laboratory in a fasting state, clad in bathrobes and slippers. On their arrival they disrobed immediately and lay upon the beds. As a further precaution to ensure similarity of conditions between the two groups successive pairs of subjects occupied alternate beds in the experimental room. Immediately after recumbency and at every half hour thereafter from 9.00 A.M. to 12.00 M. measurements were taken of the skin, oral and rectal temperatures and the oxygen consumption rates. Thus, seven sets of readings were taken each morning, each of these repetitions being designated as a 'period.' The skin temperature was measured at nineteen points, each stamped by an inked circle 0.5 inch in diameter in order to be sure that the same spot was measured on subsequent examinations, as slight variations in localization have been found to show marked differences in temperature (Freeman and Linder, '34). The areas measured are shown in figure 1.

The effects of various environmental conditions were studied. The number of measurements of skin temperature obtained in these studies has been so great that the desirability of utilizing only those readings pertaining to one side of the body was considered. This reduction of the data would be permissible only if a rigid analysis revealed a high degree of consistency between the temperatures of the two sides. This paper presents the results of such an analysis.

A similarity of the skin temperatures of symmetrically located areas of the body has been noted by several investigators (Cobet, '26; Talbot, '31). However, the actual degree of similarity has not been determined under controlled environmental conditions and with rigid attention to quantification. The results of such a study should prove interesting not only from the viewpoint of human physiology but also from that of pathology.

In the present investigation, after ten pairs of subjects had been studied, the experiment was interrupted for 6 weeks and then resumed. The average ward temperature, i.e., pre-test environment to which the second ten pairs were exposed, was significantly higher than that for the first ten pairs (21.1°C. versus 18.9°C.). The average air velocity in the experimental
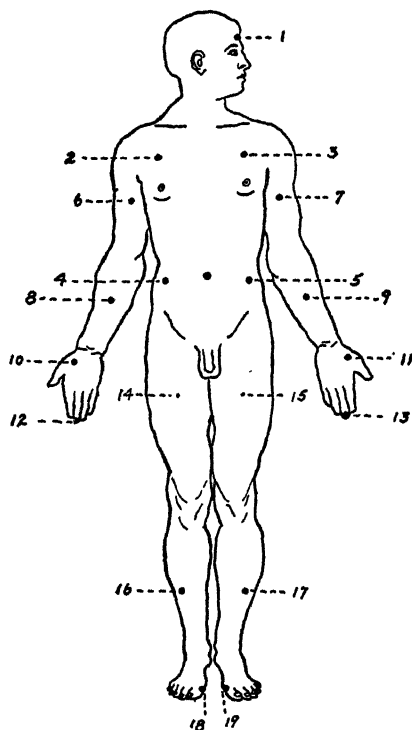
Figure 1

room was also significantly greater during the second series (40.7 feet per minute versus 22.5 feet per minute). In both series, however, the dry bulb temperature was maintained at 24°C. and the relative humidity at 20%. In view of the possible influence of these changes on skin temperature the data have been analyzed separately for each group of ten and will be so presented.

The average differences between the right and left sides for the nine locations are shown in table 1.[1] Although these differences are rather small, two-thirds of them are statistically significant, i.e., are beyond the probability of chance occurrence. They are indicative of the fact that in some areas the right side is consistently warmer and in others the left. The consistency of the signs of the differences of the two groups of controls as well as patients lends additional weight to their significance. Over the trunk, seven out of the eight differences show the left side to have the higher temperature; on the arm and hand the right side is higher in all of the sixteen instances. It would seem then, that in both the normal control and schizophrenic subjects there is a systematic variation in the average temperature difference between the two sides over the various portions of the body surface. The trunk is warmer on the left side, the arm and hand on the right, the thigh on the left and the lower leg and foot on the right. It is of interest that in a small series of normal subjects Heiser and Cohen ('33) found, from a comparison of the wrist and ankle skin temperatures that those on the right were signficantly higher than those on the left side. The explanation for this systematic variation is uncertain. It may possibly be ascribed to anatomic differences over the trunk and to the relative dominance of the right limbs. It cannot be ascribed definitely to 'handedness' for in the twenty patients only one was left handed and yet eight had lower temperatures on the right hand. Notwithstanding the significance of these differences, their relatively small magnitudes suggest a rather high degree of similarity between the temperatures of the two sides.

In addition to this description of mean differences knowledge is required of the variation that takes place between sides from individual to individual, from period to period, and of the net variation within the individual.

Values descriptive of the variation of the differences between sides from individual to individual are given in table 2

[1] Each of these average differences is based on seventy separate differences.

TABLE 1

Average differences in skin temperature (°C.) between the right and left sides in nine symmetrically located areas for seven sets of readings in twenty normal controls and twenty schizophrenic subjects (the average temperature of the left side is subtracted from that of the right side

	CHEST		ABDOMEN		UPPER ARMS		LOWER ARMS		THUMBS		FINGERS		THIGHS		LOWER LEGS		FIRST TOES	
	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.
1st 10	−0.17[1]	−0.04	−0.03	−0.03	0.11[1]	0.03	0.10[1]	0.08[1]	0.05	0.12[1]	0.07	0.34[1]	−0.09[1]	−0.14[1]	−0.10[1]	0.05[1]	0.09[1]	0.00
2nd 10	−0.09[1]	−0.09[1]	−0.03	−0.05	0.09[1]	0.11[1]	0.12[1]	0.16[1]	0.11	0.47[1]	0.19[1]	0.22[1]	−0.26[1]	−0.47[1]	0.29[1]	0.06[1]	0.02	0.02

[1] Denotes magnitudes that are statistically significant. Significance has been determined by means of Fisher's 't' test ('34) using the values in table 4 as a basis of reference.

TABLE 2

Inter-individual variation (standard deviation[1]) of differences in skin temperature (°C.) between right and left sides for nine symmetrically located areas over seven sets of readings in twenty normal controls and twenty schizophrenic subjects

	CHEST		ABDOMEN		UPPER ARMS		LOWER ARMS		THUMBS		FINGERS		THIGHS		LOWER LEGS		FIRST TOES	
	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.
1st 10	0.39	0.31	0.52	0.43	0.78	0.55	0.64	0.56	0.73	0.42	0.55	0.44	0.31	0.34	0.40	0.35	0.82	0.46
2nd 10	0.60	0.45	1.00	0.36	0.85	0.60	0.64	0.67	0.68	1.12	0.72	0.91	0.43	0.50	0.65	0.42	0.84	0.44

All values are statistically significant. Significance has been established by Fisher's 'z' test, using the values of table 4 as a basis of reference.

[1] These standard deviations pertain to the variation existing among the mean temperature differences for ten individuals. They are derived from the 'Individual-Side Interaction Variance' isolated by an analysis of variance for three criteria of classification.

for each of the nine areas. There are several interesting items in this table. First, all the values are statistically highly significant. This implies that the individuals are definitely different from each other with respect to the magnitude of the difference between the two sides for any of the nine areas studied. It will be noted that the values of the second group are higher than those of the first in all but three instances. That is, the differences between individuals are exaggerated by the introduction of some factor, such as, possibly, increased air velocity. Second, the magnitude of the variation shows no systematic trend over the nine areas studied. Thus, it would seem that the inter-individual variation of the differences between right and left sides is no greater for the extremities than for the trunk. This is somewhat surprising in view of the observed fact that individuals differ more from each other in the temperature of their extremities than in the temperature of their trunks. Third, so far as the normal control and schizophrenic subjects are concerned, the psychotic individuals have larger values in thirteen of the eighteen instances. This implies that the schizophrenic group is more heterogeneous with respect to the differences between the right and left sides than is the group of normal controls.

Values in table 3 pertain to the variation of the difference between sides as displayed from period to period. These values are all quite small and thus point to a high degree of consistency of the difference between sides from period to period. Of the thirty-six values, but four are sufficiently large to have satistical significance. While the magnitude of the difference between the temperatures of the two sides may be a particular function of the individual, it is independent of the period during which the determination was made. The values in the second group are not appreciably larger than those in the first, implying that the average difference did not fluctuate any more over the seven periods of this latter series in spite of the increased air velocity. Again the extremities do not seem to be affected to any greater extent in

this regard than are any other areas. In this instance no differences were observed between the patients and the normal controls.

The two sources of variability already discussed dealt with the aggregates of seven periods for each of ten individuals (table 2); and with aggregates of ten individuals for each of seven periods (table 3), thus furnishing data as to the variation of the difference between sides from individual to individual and from period to period. It is desirable, however, to obtain an expression of the average variability within the individual for any given period. In order to arrive at this expression the following procedure is carried out: The intra-individual variation of the difference between the two sides is determined, this quantity revealing the fluctuation of the magnitude of the difference over the seven periods within the individual; then the period-to-period variation of the group (table 3) is subtracted. The resulting values are given in table 4. The small magnitude of these instantaneous measures of variability demonstrate the high degree of similarity between temperatures of symmetrically placed areas. The fact that the values in table 2 are significantly greater than these values has already given rise to the statement that the individual is more consistent with himself than with others. The values for the thumb and finger are somewhat greater than those for other points. This implies that for the individual there is less symmetry of temperatures for these two areas. The second group has higher values with but two exceptions, implying that the intra-individual consistency has been reduced by some such factor as air velocity. Finally the values for patients are somewhat higher than those for the normal controls. There is a suggestion of less consistency between the temperature of symmetrically located areas of the body surface in schizophrenics than in normal control subjects.

TABLE 3

Inter-period variation (standard deviation)[2] of differences in skin temperature (°C.) between right and left sides for nine symmetrically located areas in twenty normal controls and twenty schizophrenic subjects

	CHEST		ABDOMEN		UPPER ARMS		LOWER ARMS		THUMBS		FINGERS		THIGHS		LOWER LEGS		FIRST TOES	
	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.
1st 10	0.10	0.07	0.14	0.10	0.32[1]	0.13	0.26	0.15	0.29	0.07	0.35	0.12	0.11	0.09	0.14	0.22[1]	0.15	0.17
2nd 10	0.30	0.19	0.18	0.15	0.17	0.21	0.22	0.19	0.22	0.24	0.25	0.62	0.08	0.10	0.12	0.12	0.09	0.25[1]

[1] Denotes magnitudes that are statistically significant, as determined from the values in table 4 and the 'z' test.

[2] These standard deviations pertain to the variation existing among the mean temperature differences for seven periods. They are derived from the so-called 'Period-Side Interaction Variance' isolated by an analysis of variance for three criteria.

TABLE 4

Net intra-individual variation (standard deviation)[1] of differences in skin temperature (°C.) between right and left sides for nine symmetrically located areas in twenty normal controls and twenty schizophrenic subjects

	CHEST		ABDOMEN		UPPER ARMS		LOWER ARMS		THUMBS		FINGERS		THIGHS		LOWER LEGS		FIRST TOES	
	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.	Pts.	N.C.
1st 10	0.09	0.09	0.10	0.09	0.16	0.11	0.15	0.13	0.24	0.25	0.33	0.30	0.10	0.08	0.11	0.11	0.18	0.07
2nd 10	0.21	0.14	0.15	0.11	0.19	0.23	0.21	0.21	0.26	0.24	0.38	0.43	0.13	0.12	0.15	0.17	0.15	0.12

[1] These standard deviations pertain to the variation existing within the individual after the period to period variation has been removed. They are derived from the so-called 'Period-Side-Individual Interaction Variance' isolated by a three-way analysis of variance.

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SUMMARY

As a part of a biological analysis of adaptative functions in schizophrenia, a study was made of the differences in skin temperature between the right and left sides in nine symmetrically located areas on the body surface of twenty normal and twenty schizophrenic individuals. Seven readings were made on each point at 30-minute intervals with the environmental temperature held at 24°C. and the relative humidity at 20%. It was found that:

1. In both normal and psychotic individuals the trunk on the left and the extremities on the right have the higher skin temperatures.

2. Individuals differ significantly from each other relative to the magnitude of the differences between the temperatures of symmetrically located areas. In this respect, however, schizophrenics differ from each other more than do normal individuals.

3. From period to period the consistency of the magnitudes of the differences between the temperatures of the two sides is very high despite the fact that the levels of temperature may be varying.

4. The variation of the magnitude of the differences between the two sides within the individual for any particular period is very small. In this respect also, the schizophrenic patient shows less consistency between the temperatures of symmetrically located areas than does the normal subject.

5. On the whole it may be safely concluded that the degree of bilateral symmetry of skin temperatures warrants the limitation of the analysis of such data to one side of the body.

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AN IMPROVED TECHNIC FOR METABOLISM STUDIES IN PRE-SCHOOL CHILDREN WITH A STATISTICAL DETERMINATION OF ITS RELIABILITY [1]

JEAN E. HAWKS, MARIE DYE AND MERLE M. BRAY

Section of Home Economics, Michigan Agricultural Experiment Station, East Lansing

ONE FIGURE

To obtain more information concerning the factors which influence the nitrogen, calorie and mineral utilization of pre-school children, the investigators in this laboratory started a series of metabolism studies in 1928. Since the quantity of protein required for optimum nutrition is one of the major diet problems, they began the study from that angle. After some preliminary balance studies, they conducted two long-time experiments on pre-school children. There were several purposes for these investigations; first, to find the variations in the nitrogen, calorie and mineral utilization of pre-school children who received a constant diet containing 3 gm. of protein per kilogram of body weight; second, to discover the changes in their metabolism when the protein content of their diet was increased to 4 gm. per kilogram; and third, to find the interrelationships between the utilizations of the various substances.

TECHNIC FOR METABOLISM STUDY

Since the technic used in any balance study largely determines the value of the results, the investigators planned the procedure and controlled the conditions very carefully. The methods used varied somewhat from those of other investigators and they may suggest some improvements in technic. Thus, it seems necessary to present the procedure in some detail before reporting the results.

Although it is impossible to control all physiological differences in individuals, the subjects are more likely to be in somewhat the same condition if they are approximately the same age and if they have lived under similar conditions for a period of time. The two boys, who served as subjects in the first experiment in this study, were each $4\frac{1}{2}$ years of age and had lived practically all of their lives in an orphanage where the food and general habits of the children were carefully controlled. In the second experiment, the two boys were 4 and $4\frac{1}{2}$ years and the two girls were each 3 years of age. These four children had all lived in boarding homes under normal conditions. Physical examination showed that all of the children were in good condition. Their weights, as shown in table 1, were similar to the weight of an average child of the same height and age (Woodbury, '23). The table also indicates that, with the exception of E, they all gained both in height and weight during the time of the experiment. E's failure to gain may be explained by the fact that at the beginning of the medium protein diet he contracted a throat infection and was quite ill for several days. Although he received the medium protein diet for 13 days at the end of the first experimental period, it did not seem wise to include any of those results, but only those from the high protein diet. Thus, his weight record is much shorter than that of the other children. All of the children represented average, normal but not superior children.

It is also impossible to control entirely the psychological effects which a balance study will have on the individual child. During the time of the study the children lived in an apart-

ment in the Home Economics building and were under constant supervision. They followed a definite routine as to play, rest, eating, toilet habits, etc. The children in the first experiment attended the college nursery school, but, on account of an epidemic of colds which occurred, those on the second experiment played by themselves. They all seemed happy and apparently did not miss their former associates.

TABLE 1

Physical measurements of children at beginning and end of experiment

SUBJECT	EXPERIMENT	AGE	HEIGHT	WEIGHT	VARIATION FROM AVERAGE WEIGHT
		months	*cm.*	*kg.*	*%*
D	I	57	108.4	17.2	—2.6
		59	109.7	17.5	—3.1
B	I	55	109.7	18.6	+2.5
		57	111.0	19.1	+2.4
J	II	38	95.3	13.6	—4.3
		40	96.5	13.9	—4.8
C	II	37	98.6	14.5	—2.0
		39	99.1	14.8	—1.3
V	II	49	102.9	16.9	+5.5
		51	103.9	17.1	+3.2
E	II	57	110.0	18.3	+0.6
		58	110.0	18.3	+0.6

The time of the study was sufficiently long to indicate the general trends in the metabolism of these children and also to show 3-day period variations. In the first experiment, they received a diet containing 3 gm. of protein per kilogram of body weight for a preliminary period of 12 and a collection period of 21 days and a diet containing 4 gm. of protein per kilogram for the following 15 days. In the second experiment, the 3-gm. protein diet continued for 10 preliminary and 21 collection days and the 4-gm. protein diet lasted for the next 24 days.

The plan of the diets removed a number of technical errors which may occur in any balance study. The results for individual children were comparable because every child received an amount of each food proportional to his body weight and thus, the same number of calories and grams of each mineral constituent per kilogram. For example, all of the children did not have the same total amount of milk, egg or other foods, but they received practically the same quantity per kilogram. If the children had been undernourished or obese, the theoretical weight would probably have been a more ideal measurement upon which to base the food needs, but the theoretical and actual weights of these children were practically the same. Since Daniels and her associates ('35) found more comparable results for nitrogen retention based on creatinine excretion, which they believe measures muscle need, it may be that calculations should have been based on this measurement. Creatinine excretion, however, would not indicate mineral needs.

The differences in the results on the medium and high protein diets would probably be due to the change in protein content because the other factors in the two diets remained fairly constant. Table 2 gives the four diets in grams of food for a child weighing 15 kg. In the first experiment, the medium and high protein diets contained the same foods with the exception of skimmed milk. Although the grams of each food were not always identical, the variations were slight, except that additional milk, ground beef and egg raised the protein content. These changes increased the percentage of animal protein from 74.2 to 82.7 and the percentage of total calories from protein sources from 14.1 to 18.5. The distribution of calories from fat and carbohydrate were quite similar, 35.0 and 50.9% in the first diet and 33.7 and 47.8% in the second. The alterations in the quantities of fruit and vegetable gave 12.6 extra gm. to the second diet, which did not radically change the bulk content. Since the mineral constituents did vary to some extent, the acid-base relationship of the diets, calculated according to the method of Salter and

his associates ('31), changed slightly. The excess of base over acid in the medium protein diet was 8.4 cc. of N solution greater than that on the high protein diet. In the second experiment, the diets were identical, except for the increase in protein content by means of egg white and gelatin, which

TABLE 2

Diet calculations for a child weighing 15 kg.

FOOD	FIRST EXPERIMENT		SECOND EXPERIMENT	
	Medium protein	High protein	Medium protein	High protein
	gm.	*gm.*	*gm.*	*gm.*
Milk	607.7	358.7	647.4	647.4
Milk (skimmed)	....	358.7	....	....
Ground beef	38.0	76.0	32.4	32.4
Egg (whole)	38.0	76.0	32.4	32.4
Egg (white)	....	....	....	64.7
Gelatin	....	....	....	8.1
Farina	15.2	15.2	....	....
Ralston's	....	....	16.2	16.2
Bread	60.8	50.6	48.6	48.6
Potato	53.2	42.2	64.7	64.7
Carrots	60.8	59.1	....	....
String beans	....	....	80.9	80.9
Tomato	76.0	84.4	80.9	80.9
Lettuce	11.8	11.8	....	....
Celery	....	....	16.2	16.2
Applesauce	76.0	76.0	121.4	121.4
Prunes	76.0	76.0	....	....
Peaches	....	....	121.4	121.4
Orange juice	151.9	168.8	161.8	161.8
Butter	15.2	16.9	16.2	8.1
Sugar	15.2	8.4	16.2	16.2
Cod liver oil	3.8	3.8	....	....
Haliver oil (250D)	....	....	1 pearl	1 pearl
Total fruit and vegetable	505.7	518.3	647.3	647.3
Protein	45.0	61.1	45.0	60.3
Animal source (%)	74.2	82.7	73.0	79.8
Calories	1282.0	1319.0	1351.0	1352.0
Protein (%)	14.1	18.5	13.3	17.8
Fat (%)	35.0	33.7	31.9	27.4
Carbohydrate (%)	50.9	47.8	54.8	54.8
Calcium	0.912	1.081	0.965	0.973
Phosphorus	1.032	1.282	1.031	1.039
Excess base over acid (cc. N.)	41.2	32.8	32.4	28.3

contained practically nothing but protein, and the omission of an equivalent number of calories from butter, which kept the calorie content of the diets at the same level. Since the extra protein was from animal source, the percentage of animal protein increased from 73.0 to 79.8. The distribution of calories from protein, fat and carbohydrate was 13.3, 31.9 and 54.8% on the first diet and 17.8, 27.4 and 54.8% on the second. Since the mineral content was practically identical, the acid-base relationship changed only 4.1 cc. N solution.

Although the second experiment was slightly more constant than the first, the results would certainly be comparable. There were a few differences in the foods served. In the second experiment, the children preferred Ralston's, string beans and celery to farina, carrots and lettuce. On account of the organic acids formed, peaches replaced prunes and because it was easier to feed quantitatively, haliver oil 250D took the place of cod liver oil.[2] Although the grams of the same foods were not identical in the two experiments, the organic and inorganic constituents were quite similar. In both experiments, the children had a measured quantity of distilled water for drinking.

The preparation and feeding of the food eliminated many of the possible errors. To reduce the probable variations in the composition of the same food, a quantity of canned and dried foods sufficient to last for the entire experiment and enough perishable foods to last for a 3-day period were purchased at one time. Great care was observed in preparing duplicate samples of food for analysis which would be identical with the portions fed to the children. The entire quantity of each food required for a 3-day period was made into a homogeneous mixture. The fat and gristle was removed from meat and the lean portions ground, eggs were beaten, potatoes were cooked and mashed and vegetables and fruits were pureéd. All samples of one food were weighed at the same

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[2] The haliver oil was supplied by Parke, Davis and Company, Detroit, Michigan, and the cod liver oil was obtained from E. L. Patch and Company, Boston, Massachusetts.

time in order to avoid changes due to water loss. Losses due to transferring food were avoided by weighing the portions for the children into the same utensils in which they were later cooked and served and by rinsing the samples for analysis into large enamel bowls with distilled water. In order to eliminate spoilage, the egg and meat dishes for the children were heated on the first day and all of the food was stored in an ice box at a low temperature. The samples for analysis were partially dried on a steam bath and then left in a constant temperature oven at 60°C. until they no longer lost weight. They were then ground several times and put through a fine-meshed sieve, the hard portions being mortared before the final sifting, when they were stored for analysis.

The food consumption was as nearly quantitative as possible. The children ate all of their meals under the constant supervision of a tactful helper who taught them to be very careful not to spill their food. She scraped the dishes carefully, wiped them out with a small piece of bread, which the children ate, and finally rinsed them with part of the child's allotted distilled water which she added to another food that the child had not eaten.

The collection and preservation of urine and feces samples were as accurate as possible. To prevent loss of urine samples, the younger children followed a definite schedule as to hours of collection. If, however, an accident occurred, the complete 24-hour sample was discarded and the results for the other 2 days in the period averaged. To lessen decomposition, samples were kept in an ice box at all times until they were complete. After the sample was measured and the specific gravity taken, it was made up to a definite volume with redistilled water. The nitrogenous constituents were determined daily and an aliquot portion of each day's sample was combined into a 3-day composite for mineral determinations. These samples were dried on a steam bath, ashed at a low temperature in platinum dishes, dissolved in 5 to 10% HCl, made up to 100 cc. and saved for analyses. In the first experiment, feces were collected in daily samples, charcoal

being used as a marker one day and carmine the next. A portion of the moist feces was partially digested into 20% H_2SO_4 and Kjeldahl determinations made on this sample. The remainder of the feces was dried. In the second experiment, feces were marked with carmine, collected in 3-day periods, prepared in the same manner as the food and stored until used.

Before each chemical determination, care was taken to obtain a homogeneous sample having the same moisture content as the original dried food. In order to do this the ground food was sifted, thoroughly mixed and a portion of it dried overnight in a constant temperature oven at 60°C. From this sample, triplicate portions were weighed for the estimation of both calories and nitrogen. Calories were determined in an oxy-calorimeter as described by Benedict and Fox ('25 a, '25 b) and nitrogen was estimated by the official Kjeldahl method (Association of Official Agricultural Chemists, '25). For minerals, duplicate samples varying in weight from 15 to 20 gm. were ashed in platinum dishes at a temperature below red heat, dissolved in hydrochloric acid and made up to 100 cc. Calcium was estimated according to the method described by Kramer and Howland ('26) except that the precipitated calcium oxalate was filtered through a porous bottom crucible rather than filter paper. Phosphorus was determined by the uranium titration as outlined by Peters and Van Slyke ('32). In a few cases in which the results did not check within 2%, a third ashing was made and all three results averaged.

STATISTICAL EVALUATION OF THE CONSTANCY OF THE DIET COMPOSITION

Diet calculations from standard tables of food composition give only approximate values, since the composition of individual food varies with a number of factors, such as variety of the product, degree of freshness and the type of soil, climatic conditions and season of the year in which it was grown. Therefore, it is necessary to analyze the food used in order to obtain the actual composition.

There are variations in the composition of identical diets even when the experimental conditions are carefully controlled. Porter-Levin ('33) found that the calcium and phosphorus intakes of her subjects, who received the same diet during a number of periods, varied within fairly small limits. The coefficients of variation were between 2 and 3. Bassett and Van Alstine ('35) obtained similar coefficients for the nitrogen content of identical diets, but higher values, from

TABLE 3

Range and variability in composition of identical diets

ELEMENT	EXPERI- MENT	PROTEIN PER KILO- GRAM IN DIET	NUMBER OF SAMPLES	COMPOSITION		STANDARD DEVIATION	COEFFI- CIENT OF VARI- ATION
				Range	Mean		
		gm.					%
Calories	I	3	18	1444–1558	1497± 4.7	29	2.0
		4	8	1543–1677	1596±10.2	43	2.7
	II	3	16	1132–1219	1171± 3.9	23	2.0
		4	16	1128–1199	1164± 3.8	23	1.9
Nitrogen (gm.)	I	3	18	8.40– 9.19	8.77±0.04	0.24	2.7
		4	10	11.18–12.15	11.85±0.06	0.27	2.3
	II	3	16	5.62– 6.28	6.01±0.03	0.18	3.0
		4	16	7.93– 8.37	8.19±0.02	0.13	1.6
Calcium (mg.)	I	3	18	987–1076	1032±3.8	24	2.3
		4	10	1173–1233	1200±4.0	19	1.6
	II	3	16	716– 793	755±3.9	23	3.1
		4	16	736– 831	776±4.1	24	3.1
Phosphorus (mg.)	I	3	18	1097–1194	1143±4.4	28	2.4
		4	10	1405–1507	1456±8.2	38	2.6
	II	3	16	825– 895	863±3.8	22	2.6
		4	16	833– 915	874±3.2	19	2.2

3.0 to 8.8, for calcium and phosphorus. In the present study the calorie, nitrogen, calcium and phosphorus analyses of identical diets and of duplicate diets collected from the same food on the same day give some further information concerning variability in composition.

Table 3 presents the range in the calorie, nitrogen, calcium and phosphorus values which were obtained by the analyses of from eight to eighteen identical samples of the four different diets. Since Dunn ('29) has shown that statistical

methods may be applied to a small series of analyses, the table also gives the mean with its probable error, the standard deviation and the coefficient of variation. These constants were calculated according to the following formulae:

$$M = \frac{\Sigma(x)}{n}$$

$$\text{P.E. of } M = 0.6745 \left(\frac{\sigma}{\sqrt{n}}\right)$$

$$\text{S.D. or } \sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$$

$$\text{C.V.} = 100 \left(\frac{\sigma}{m}\right)$$

The coefficients of variation, which represent the relative extent to which the standard deviations vary from the means, were quite constant for all constituents analyzed in each series of determinations. These values, which ranged from 1.6 to 3.1, showed that although the identical diets did vary somewhat in their composition, the quantity of the different elements varied in about the same degree. Nitrogen figures displayed variations similar to those which Bassett and Van Alstine ('35) reported. The values for calcium and phosphorus, on the other hand, did not show as large variations as these authors found, but fluctuated in about the same manner as the figures which Porter-Levin ('33) gave.

There are several factors which may have caused these variations in composition. In the first place, there were the unavoidable errors in chemical technic. These errors may have produced a large proportion of the discrepancies, because Bassett and Van Alstine ('35) found that the coefficients of variation in the results of a number of consecutive analyses of a single specimen were 0.96 for calcium and 1.61 for phosphorus. In the second place, it was apparently impossible to obtain a homogeneous sample of food for analysis, even when conditions were carefully controlled. Table 4 shows that the composition of duplicate diets collected in the same manner from the same food on the first day of the period varied as much as 6.7% and the average differences ranged from 1.0 to 3.2%. Figure 1 also illustrates the variations between the two analyses on the same period.

In all probability a third factor, the variation in the composition of the same food, also influenced the results because analyzed and calculated values differ. Table 4 shows that the analyzed values in this study varied from the calculated figures as much as 7.3% for calories, 9.4% for protein, 13.9% for calcium and 10.3% for phosphorus but that the average

TABLE 4

Percentage variability in composition of diets

ELEMENT	EXPERIMENT	PROTEIN PER KILOGRAM IN DIET	DIFFERENCE BETWEEN DUPLICATE DIETS		VARIATIONS FROM CALCULATED VALUES		VARIATION OF EACH VALUE FROM MEAN		VARIATION OF PERIOD AVERAGE FROM MEAN		
			Range	Av.	Range	Av.	Range	Av.	Range	Av.	Coefficient of variation
		gm.	%	%	%	%	%	%	%	%	%
Calories	I	3	0.8–4.3	1.8	0.0–4.9	1.9	0.2–4.1	1.6	0.1–3.3	1.3	1.6
		4	0.1–4.0	1.3	1.2–7.3	2.7	0.2–5.1	1.9	0.4–3.3	1.9	2.2
	II	3	0.3–3.6	1.7	0.2–4.5	1.6	0.2–4.1	1.6	0.3–2.9	1.4	1.8
		4	0.2–2.3	1.0	0.2–3.3	1.6	0.1–3.1	1.6	0.3–2.9	1.6	1.9
Nitrogen	I	3	0.5–4.6	2.0	0.0–7.8	3.1	0.0–4.9	2.1	0.1–3.3	2.0	2.3
		4	0.3–4.8	1.7	1.2–4.9	3.0	0.0–5.7	1.5	0.2–3.4	1.5	2.0
	II	3	0.0–4.6	2.0	0.1–9.4	3.4	0.5–6.3	2.5	0.1–4.1	2.2	2.8
		4	0.7–3.8	2.0	0.1–4.8	1.8	0.0–3.2	1.3	0.1–2.5	0.9	1.2
Calcium	I	3	0.5–3.9	1.9	0.5–8.7	4.5	0.0–4.4	1.7	0.3–4.1	1.6	2.0
		4	0.7–2.5	1.4	3.7–8.4	6.3	0.1–2.8	1.2	0.4–2.3	1.1	1.4
	II	3	0.1–4.3	2.7	4.7–13.9	9.3	0.0–5.2	2.3	0.4–4.5	2.2	2.8
		4	0.3–6.3	3.2	1.0–12.3	7.5	0.0–7.1	2.3	0.5–4.6	2.0	2.5
Phosphorus	I	3	0.3–6.7	2.4	2.4–10.3	6.6	0.1–4.5	1.9	0.3–3.0	1.6	1.9
		4	0.4–3.8	2.3	0.8–7.5	4.2	0.1–3.5	2.2	0.0–3.3	1.8	2.4
	II	3	1.4–5.3	2.8	0.1–7.5	3.2	0.2–4.4	2.2	0.8–3.0	1.9	2.2
		4	1.3–3.8	2.3	0.3–7.3	3.0	0.1–4.7	1.6	0.1–3.0	1.4	1.8

variations were small. It is not surprising that the percentage differences were, in the majority of cases, lower than those which Bray, Hawks and Dye ('34) obtained because their data included many cooked foods the raw weight composition of which was not known. The reason that the differences were lower than those which Bassett and Van Alstine ('35) and Donelson and her associates ('31) obtained, may have been due to the fact that although the average data given by Rose ('32) formed the basis of the calculated values, the
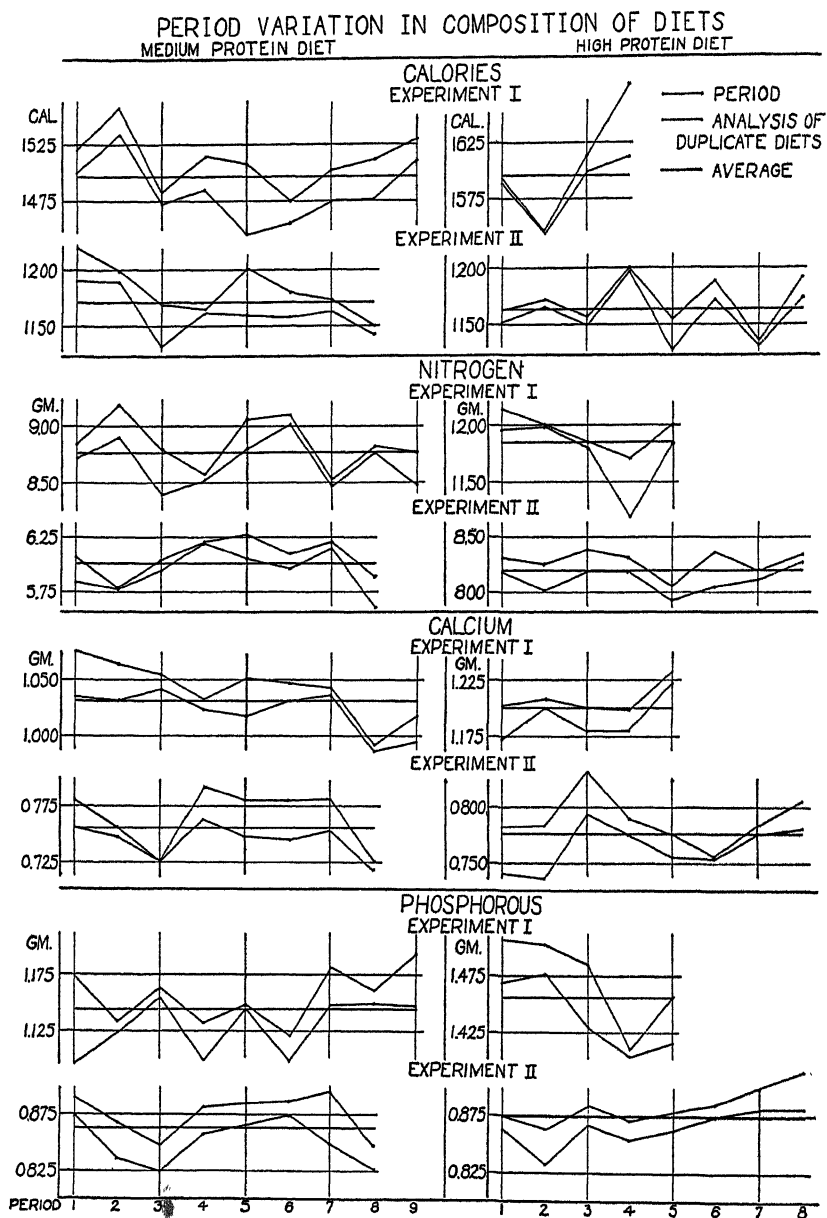
Figure 1

figures for canned pureéd fruits and vegetables were actual analyses. The variation in the composition of the same food was also illustrated by the fact that there was less difference between the composition of duplicate diets collected from the same food than there was between the highest and lowest value for the eighteen identical diets analyzed (fig. 1). In order to show this difference mathematically, table 4 expresses the variation of all the data from the mean in terms of per cent. The highest of these values, 7.1%, which represents just half of the difference between the highest and the lowest value, was practically the same as the greatest total difference between the composition of the duplicate diets, 6.7%. Therefore, the analysis of any one sample of diet may vary from that of any other sample about twice as much as the difference between the analysis of two samples of the same diet.

Since the values for the duplicate diets usually varied in the same direction from the mean (fig. 1) and showed a period to period variation, the average of the two diets may represent the composition of the food which the children actually received more accurately than either of the separate analyses. Nevertheless, the food which they received may have varied as much as the difference between the duplicate diets or approximately 3.4% from the period average. This period average showed less variation than the values for the individual analyses because the greatest percentage difference from the average of all the data was only 4.6 (table 4). Although the number of cases for statistical treatment were considerably reduced, the coefficients of variation were lowered to values between 1.2 and 2.8.

The variation in the composition of the food is an unavoidable part of every metabolism study, and it seems probable that the metabolic responses of the subjects would reflect these variations in diet. Since the differences would not be the same in successive experiments in one laboratory or in experiments in different laboratories, it is probably necessary to determine the fluctuations in every experiment.

SUMMARY

The procedure used in two balance experiments conducted in this laboratory is described. Some of the precautions taken may suggest improvements in technic which might eliminate some technical errors and make results more constant.

The composition of diets containing the same food and prepared under standard conditions varied not only from period to period but also between duplicate diets collected on the same day. Nevertheless, the coefficients of variation for calories, nitrogen, calcium and phosphorus were not large, ranging between 1.6 and 3.1.

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THE IRON METABOLISM OF NORMAL YOUNG WOMEN DURING CONSECUTIVE MENSTRUAL CYCLES [1]

RUTH M. LEVERTON AND LYDIA J. ROBERTS

Department of Home Economics and Household Administration, The University of Chicago

THREE FIGURES

Information concerning the quantitative metabolism of iron in normal women is meager. A search through the literature yields little in regard to the interrelation of blood formation, menstruation, and iron retention. The difficulties of analysis of iron in biological materials, the small amount of iron metabolized by the human organism, and the variation in the physiological state of the subject as a result of the menstrual process have all been reasons for the scarcity of research in this field.

Two studies of the iron metabolism of normal women over short periods of time have been reported in the last 2 years. Ohlson and Daum ('35) studied the iron exchanges of three healthy young women under normal conditions of living and at intervals over $2\frac{1}{2}$ months. Complete collections were made during six periods which ranged from 5 to 15 days in length and included at least one menstrual period for each subject. The diets contained "liberal amounts of protein, calories and protective elements." The average daily intake of iron for all periods was 13.78 mg. and the average daily excretion

65

was 14.95 mg. The losses in the menstrual hemorrhage were 25.68 and 41.90 mg. for two subjects and 18.16 and 32.35 mg. for two different periods for the third subject. The authors report that there was no relationship between iron retention and the losses during menstruation. Because their observations were limited to isolated portions of the menstrual cycles of the subjects rather than including complete ones the metabolic picture in regard to iron exchanges is incomplete.

The same is true of the study of Farrar and Goldhamer ('35). One subject, a dietitian, lived on a diet which furnished 9.1 mg. of iron daily for 41 days. The average daily intake for 17 days during the intramenstrual phase was 8.3 mg. and the excretion 8.2 mg. Approximately 33 cc. of blood were lost in menstruation. The balance was not given for the rest of the experimental time.

A complete study of the iron metabolism of women involves certain difficulties. Because 70% of the iron in the body is present in the blood (Sherman, '33) a complete iron balance must consider in so far as possible all blood losses. These may be due to the bleeding of a cut finger, a nosebleed, drawing venous blood for routine analysis, and most particularly to menstruation. Having included these additional paths of possible loss of iron the question arises in calculating a balance of iron as to how these losses should be considered. It may be normal for a woman to be in negative balance during the few days of menstruation and in positive balance at some later time to compensate for this loss or to prepare for the next loss. The results of a brief iron balance study on a woman might depend therefore on the relation of the time of the study to her menstrual period. However, this variable could be obviated by determining her exchanges of iron over a period of time which would include all the changes related to the menstrual process. Thus a menstrual cycle—the recurring period during which the body, particularly the mucous membrane of the uterus, passes through all the changes associated with menstruation—appears to be the most logical time unit for a complete study of the iron balance of women. Also because of the

recognized variations inherent in daily metabolism, figures for the total balance during a cycle would be more significant than calculations for average daily balances.

In view of these facts the present investigation was planned for the purpose of observing by means of a continuous controlled balance experiment the total iron exchanges of normal women during consecutive menstrual cycles. It is hoped that interpretation of the data obtained will contribute to the knowledge of the iron requirement of women, the degree of the iron losses during menstruation, the time and extent of the storage of iron following these periodic losses, and the hemoglobin and red cell content of the blood during different phases of the menstrual cycle.

EXPERIMENTAL PROCEDURES

The investigation constituted a balance study which involved the weighing and analyzing of all the food eaten by the subjects and the collection and analysis of all the excreta during the experiment. Beginning January 2, 1935, the study extended continuously over a period of 110 days for two of the subjects and 140 days for the other two subjects. Four healthy young college women served as subjects both for this study and the study of calcium and phosphorus metabolism which was being conducted at the same time. Three of them were 21 years old and were carrying an ordinary schedule of college work. The fourth one was 27 years old and had a strenuous schedule of laboratory work in connection with this metabolism experiment. Determinations of basal metabolic rate which were made on all subjects 3 consecutive days at the beginning and at the end of the study showed them to be normal in this respect. The menstrual process was reasonably regular in all the subjects and normal according to the subjective criteria of amount and duration of flow.

Daily records were kept of each subject's weight, taken without clothing and before breakfast, of the number of hours of sleep, of any unusual exercise engaged in, and of definite emotional or physical disturbances during the study. Daily

determinations of hemoglobin and red blood cells were made during the greater part of the study and the results are reported in a separate article (Leverton and Roberts, '36). The amount of venous blood which was taken twice during each menstrual cycle in connection with the calcium and phosphorus study was recorded so that the iron lost in this way might be included in the final iron balances.

Planning the dietary for this prolonged metabolic study presented a real problem. One of the purposes of the experiment was to study normal iron metabolism in several subjects when there was constant intake of this mineral from dietary sources over a long period of time. However, directly opposed to the theoretical attainment of this situation was the necessity of adjusting the caloric intake to the energy requirement of each subject, of satisfying individual food likes, and of offering enough variety to make it possible for the subjects to be content to live on this diet with never a break in routine for as long as 5 months. In addition to this was the knowledge of the notorious lack of uniformity in the composition of foods available on the retail market and the problem of securing and keeping the foods from a uniform source of supply. In an attempt to reconcile these conflicting factors without sacrificing too much from the standpoint of having a constant intake nor from the standpoint of having a long-time study the total time of the experiment was divided arbitrarily into consecutive 5-day periods. Then a single foundation dietary was planned and used for every subject during every 5-day period. By varying the order and combination of the different foods during a period a wide variety in the menus could be furnished but the total intake of the foundation dietary was still almost identical in kind and amount for all subjects for all periods. In addition to the main diet certain foods chosen because of their low mineral content were permitted ad libitum. The psychological value of this practice was found to be immeasurable. A list of the kinds and amounts of foods used is given in table 1. During the fourteenth to the twenty-second periods subject A received a daily supplement of 5 mg. of iron in the

form of ferric ammonium citrate, and as part of the calcium and phosphorus study subjects C and D received 353 Steenbock units of vitamin D from biologically standardized cod liver oil daily.

Every attempt was made to secure foods of uniform composition. The canned goods were bought in case lots and except when extremely impractical the perishable foods were bought from the same source throughout the 5 months. All the food was prepared and served in one of the nutrition

TABLE 1

Individual dietary for each 5-day period

FOODS FOUNDATION DIET	AMOUNT IN GRAMS	FOODS FOUNDATION DIET	AMOUNT IN GRAMS	FOODS AD LIBITUM
Orange juice	200	Lettuce	50	White bread
Orance ice	100	Onions	60	Butter
Tomato juice	200	Peas, canned	70	Jelly
Tomato soup concentrate	70	Potatoes, white	200–400	Apples
Grapefruit	100	Potatoes, canned sweet	100	Salad dressing
Peaches, canned	80	Whole wheat bread	300	Jello
Pineapple, canned	80	Doughnuts	60	Plain cookies
Banana	100	Bacon	20	Candy
String beans, canned	70	Beef, lean	400	Coffee
Beets, canned	70	Salmon	90	Tea
Cabbage	100	Eggs, whole	150	Distilled water
Carrots	100	Milk	2400	

laboratories. Since the plan of this study was to approximate an ordinary situation of food preparation and consumption no attempt was made to protect the food from contamination with iron before it was served. Enamel and aluminum ware utensils were used for cooking and although distilled water was used instead of tap water it was only because the subjects drank different amounts and the tap water was known to vary in its mineral content. However, as soon as the food was served to the subjects a portion was placed in a screw top glass jar and then every precaution was taken to keep this sample from collecting additional iron from the air, utensils or containers. This portion was used for making the daily

food composites which represented one-tenth of the amount of all food eaten by each subject. The five daily composites were combined to form a period composite for each subject and this was analyzed for iron.

After the subjects had been on the experimental diet for 10 days, collection of the excreta was begun. All collections of urine and feces were made directly into glass containers. Carmine was given to mark the beginning of each period. All stools for each subject during each 5-day period were combined into a period fecal composite and aliquot portions of daily urine collections were combined into a period urinary composite. The menses were collected on a standard brand of cellulose pad from a single lot which had been sampled at random for blank analysis.

The food and fecal composites and the collection of each menstrual flow were made into smooth brown digests with sulfuric acid by the method of Stearns ('29) with modifications necessary to make it applicable to material which was to be used for iron analyses. Instead of being boiled with the acid the material and the acid were heated at 60 to 70°C. for 2 weeks at the end of which time a homogeneous emulsion resulted. This could be stored indefinitely. The urine was acidified and stored also.

The difficulties involved in the micro-analysis of iron in biological materials are widely recognized and have received much critical attention of late. No one method seems satisfactory from every standpoint nor gives results of unfailing accuracy at the hands of all workers. In this study iron was determined as the thiocyanate by the method developed by Stugart ('31). This method with careful application and certain adaptations gave admirably accurate results. Space does not permit reporting here the results of the extensive laboratory work and the meticulous routine necessary to establish definitely the adequacy of this method. Every effort was made to prevent the contamination of the samples by iron-laden dust particles. A small protected laboratory of wood and glass was constructed within the larger chemistry laboratory in order to have a place where the samples could be

handled without their accumulating extraneous iron. The method of treatment of the samples consisted in drying and carefully ashing at about 400° C. aliquot portions of the brown digests and of the urine composites, and dissolving the ash in hydrochloric acid. Aliquot portions of the ash solution were then hydrolyzed with acid to change the pyrophosphates to orthophosphates. The iron present was converted to ferric thiocyanate and its amount determined by colorimetric comparison in amyl alcohol solution with a standard solution of iron. At first triplicate determinations were made on each of the ash solutions of three aliquots from each digest but later this number was reduced to duplicate determinations on the ash solution of each of two aliquots from each digest.

For several reasons only total iron was determined in food and excreta in this study in spite of the fact that recent research indicates that iron must exist in the inorganic form to be available for hemoglobin regeneration. Among these was the fact that the most satisfactory method for determining inorganic iron requires the use of individual samples of fresh food material and not composites in which the lack of a homogeneous mixture makes accurate sampling impossible. Daily analysis of individual samples would have been prohibitive in number because of the variety of foods used in each period and the different combinations in which they were prepared. Since the method of preparation can have such a variable effect on the inorganic iron in foods, figures for the average available iron content secured from analysis of a few samples would have been little less than approximations. Moreover, the non-availability of iron in the organic form for hemoglobin building may be due to its incomplete absorption from the intestine (Vahlteich et al., '36). The problem of absorption and re-excretion of iron by the gastro-intestinal tract is not within the realm of a balance experiment and therefore the determination of inorganic or available iron in the feces would be of no more value than one of total iron. Finally, availability has as yet only been demonstrated to apply to the utilization of iron for hemoglobin formation following very

severe anemia and since iron is an essential constituent of animal protoplasm, even though 70% of it exists as hemoglobin, it seemed more pertinent in this study of iron exchanges in normal women to determine total rather than available iron.

<center>RESULTS</center>

The data from this study of iron metabolism will be presented in the following order: 1) the balance between the average daily intake and fecal and urinary excretion of iron for each 5-day period, for each menstrual cycle, and for the entire study; 2) the iron losses in the menses; 3) the balance for each menstrual cycle including the iron lost in the menses; and 4) the actual balance for each cycle and for the entire study when the iron lost through the drawing of venous blood samples and through a nosebleed for one subject is considered.

Balance between food and excreta. The average daily intake, excretion and balance of iron of the four subjects during the consecutive 5-day periods of the metabolism study were calculated from the results of the analysis of the composites of the food, urine and the feces for each period. The results are presented in table 2 expressed in total milligrams of iron per day and in milligrams of iron per kilogram of body weight per day for each subject for each 5-day period. In this table the excretion includes both urinary and fecal iron. Because the daily quantity of iron in the urine was small (0.19, 0.17, 0.29 and 0.19 mg. for subjects A, B, C and D, respectively) and constant it seemed justifiable to add it to the many times larger fecal excretion and thus report only one figure for the excreta. Even though the daily urinary excretion of iron was not great enough to be significant in determining a daily balance such a loss could not be disregarded over a period of several months.

When the average daily amount of iron ingested is compared with the average daily amount excreted in the feces and urine for the entire metabolic period subjects A, B and C were in positive balance and subject D in slight negative balance.

TABLE 2

Average daily intake, excretion and balance of iron of four subjects during consecutive 5-day periods of the metabolism study

SUBJECT	PERIOD	AVERAGE MILLIGRAMS IRON PER DAY			AVERAGE MILLIGRAMS IRON PER KILOGRAM PER DAY		
		Intake	Excretion	Balance	Intake	Excretion	Balance
A	A	9.86	8.11	+1.75	0.166	0.137	+0.029
	B	12.08	12.83	—0.75	0.204	0.216	—0.012
	C	10.56	10.35	+0.21	0.180	0.177	+0.003
	D	10.16	13.15	—2.99	0.176	0.224	—0.048
	E	12.33	8.11	+4.22	0.210	0.138	+0.072
	F	11.36	9.79	+1.57	0.194	0.167	+0.027
	G	11.84	11.95	—0.11	0.200	0.203	—0.003
	H	14.16	12.67	+1.49	0.241	0.216	+0.025
	I	12.48	12.03	+0.45	0.212	0.205	+0.007
	J	14.00	11.47	+2.53	0.238	0.195	+0.043
	K	11.12	11.07	+0.05	0.227	0.187	+0.040
	L	13.60	12.67	+0.93	0.232	0.216	+0.016
	M	13.44	9.63	+3.81	0.230	0.164	+0.066
	N	17.68	19.39	—1.71	0.300	0.331	—0.031
	O	18.16	25.79	—7.63	0.308	0.437	—0.129
	P	18.16	16.83	+1.33	0.308	0.284	+0.024
	Q	17.04	21.59	—4.55	0.288	0.365	—0.077
	R	16.16	8.29	+7.87	0.272	0.140	+0.132
	S	16.56	14.35	+2.21	0.279	0.242	+0.037
	T	17.28	16.52	+0.76	0.290	0.277	+0.013
	U	17.52	16.92	+0.60	0.295	0.285	+0.010
	V	12.54	12.67	—0.13	0.210	0.212	—0.002
	W	11.60	12.63	—1.03	0.193	0.210	—0.017
	X	10.24	7.87	+2.37	0.170	0.132	+0.038
	Y	11.52	11.71	—0.19	0.190	0.198	—0.008
	Z	12.16	7.15	+5.01	0.204	0.120	+0.084
	Average	13.61	12.89	+0.72	0.231	0.220	+0.011
B	A	10.85	2.30	+8.55	0.180	0.037	+0.043
	B	10.64	10.39	+0.25	0.174	0.172	+0.002
	C	10.96	13.77	—2.81	0.178	0.223	—0.045
	D	10.00	12.41	—2.41	0.164	0.203	—0.039
	E	9.84	8.25	+1.59	0.163	0.136	+0.027
	F	12.56	8.81	+3.75	0.209	0.147	+0.062
	G	10.64	9.85	+0.79	0.175	0.163	+0.012
	H	14.56	11.13	+3.43	0.240	0.183	+0.057
	I	12.56	10.01	+2.55	0.208	0.165	+0.043
	J	12.44	12.89	+0.45	0.205	0.211	—0.006
	K	11.20	14.49	—3.29	0.185	0.240	—0.055
	L	12.88	9.53	+3.35	0.213	0.158	+0.055
	M	12.96	9.61	+3.35	0.214	0.159	+0.055
	N	12.00	14.01	—2.01	0.200	0.234	—0.034
	O	11.92	7.37	+4.55	0.198	0.122	+0.076
	P	13.84	10.65	+3.19	0.227	0.175	+0.052
	Q	13.04	11.45	+1.59	0.216	0.189	+0.027
	R	11.12	10.49	+0.63	0.183	0.173	+0.010
	Average	11.87	10.39	+1.48	0.196	0.172	+0.024

TABLE 2—*Continued*

SUBJECT	PERIOD	AVERAGE MILLIGRAMS IRON PER DAY			AVERAGE MILLIGRAMS IRON PER KILOGRAM PER DAY		
		Intake	Excretion	Balance	Intake	Excretion	Balance
C	A	11.12	9.01	+2.11	0.242	0.198	+0.044
	B	10.08	9.49	+0.59	0.222	0.208	+0.014
	C	10.72	8.37	+2.35	0.235	0.185	+0.050
	D	10.48	9.17	+1.31	0.230	0.201	+0.029
	E	10.72	8.35	+2.37	0.234	0.183	+0.051
	F	13.04	10.61	+2.43	0.287	0.232	+0.055
	G	9.44	11.17	—1.73	0.206	0.244	—0.038
	H	11.76	8.93	+2.83	0.255	0.194	+0.061
	I	10.48	9.57	+0.91	0.228	0.208	+0.020
	J	10.03	7.53	+2.50	0.221	0.163	+0.058
	K	9.44	7.57	+1.87	0.202	0.165	+0.037
	L	8.72	10.61	—1.89	0.190	0.231	—0.041
	M	10.16	9.49	+0.67	0.222	0.207	+0.015
	N	10.72	10.21	+0.51	0.232	0.221	+0.011
	O	11.60	11.25	+0.35	0.251	0.243	+0.008
	P	9.60	11.41	—1.81	0.212	0.251	—0.039
	Q	10.64	8.17	+2.37	0.234	0.180	+0.054
	R	8.24	7.73	+1.51	0.182	0.171	+0.011
	S	10.48	9.25	+1.23	0.233	0.205	+0.028
	T	8.48	8.53	—0.05	0.187	0.189	—0.002
	U	10.80	9.01	+1.79	0.238	0.199	+0.039
	V	10.48	10.13	+0.35	0.234	0.226	+0.008
	W	9.84	12.13	—2.29	0.221	0.273	—0.052
	X	7.92	8.69	—0.77	0.176	0.193	—0.017
	Y	8.00	6.85	+1.15	0.184	0.157	+0.027
	Z	7.76	7.89	—0.13	0.183	0.187	—0.004
	Average	10.03	9.32	+0.71	0.221	0.204	+0.017
D	D	10.16	12.43	—2.27	0.186	0.228	—0.042
	E	10.80	14.99	—4.19	0.200	0.277	—0.077
	F	10.88	11.47	—0.59	0.202	0.214	—0.012
	G	9.36	11.15	—1.79	0.173	0.206	—0.033
	H	12.08	11.31	+0.77	0.222	0.208	+0.014
	I	11.28	4.71	+6.57	0.207	0.086	+0.121
	J	11.44	21.15	— 9.71	0.209	0.387	—0.178
	K	16.16	11.95	+5.21	0.295	0.219	+0.076
	L	15.04	13.09	+1.95	0.275	0.239	+0.036
	M	12.16	12.89	—0.73	0.222	0.236	—0.014
	N	11.52	13.29	—1.77	0.209	0.241	—0.032
	O	11.76	9.43	+2.33	0.214	0.171	+0.043
	P	11.60	10.69	+0.91	0.211	0.194	+0.017
	Q	11.28	8.39	+2.89	0.206	0.153	+0.053
	R	10.16	11.71	—1.55	0.185	0.213	—0.028
	Average	11.71	11.91	—0.20	0.214	0.218	—0.004

As given in table 2 the average daily intake of iron for subjects A, B, C and D was 13.61, 11.87, 10.03 and 11.71 mg. and the average daily balance was + 0.72, + 1.48, + 0.71 and — 0.20 mg., respectively.   These balances represented 5.3, 12.5, 7.1 and 1.7% of the respective intakes.

When the data are expressed in terms of milligrams of iron per kilogram of body weight and only the averages are considered it appears that the highest retention of iron occurred on the lowest intake but when the figures for individual periods are analyzed other facts may be noted.   The average daily intakes of iron for subjects A, B, C and D were 0.231, 0.196, 0.221 and 0.214 mg., respectively, with corresponding balances of 0.011, 0.024, 0.017 and — 0.004.   In figure 1 the average daily intake of iron from food per kilogram for each subject during each period is plotted in relation to the average daily retention per kilogram which resulted on each intake.   Here it may be seen that in general whether considering all subjects for all periods or each subject for all periods the greater retentions occurred on the greater intakes.   Of the twenty-six periods during which the subjects were in negative balance, thirteen occurred when the intake was less than 0.200 mg. per kilogram and the other thirteen cases when the intake was between 0.200 and 0.225 mg. per kilogram.   Also during the fifty-two periods when the daily intake was less than 0.225 mg. a negative balance occurred in 26 or 50% of the periods.   In other words, in this study there was an even chance of the body being in positive or in negative iron balance when the intake ranged from 0.160 to 0.225 mg. per kilogram, but it is significant to note that no negative balances occurred when the intake was 0.225 mg. or more per kilogram per day.

Observation of these data shows the occurrence of many variations in both intake and output of iron.   The extent and significance of these variations are shown in table 3.   As might be expected the variability of the iron in the food is much less than that in the excreta.   The standard deviations for iron intakes range from 1.20 to 1.65 with an average of 1.35; those for iron excretions from 1.30 to 4.34 with an average of
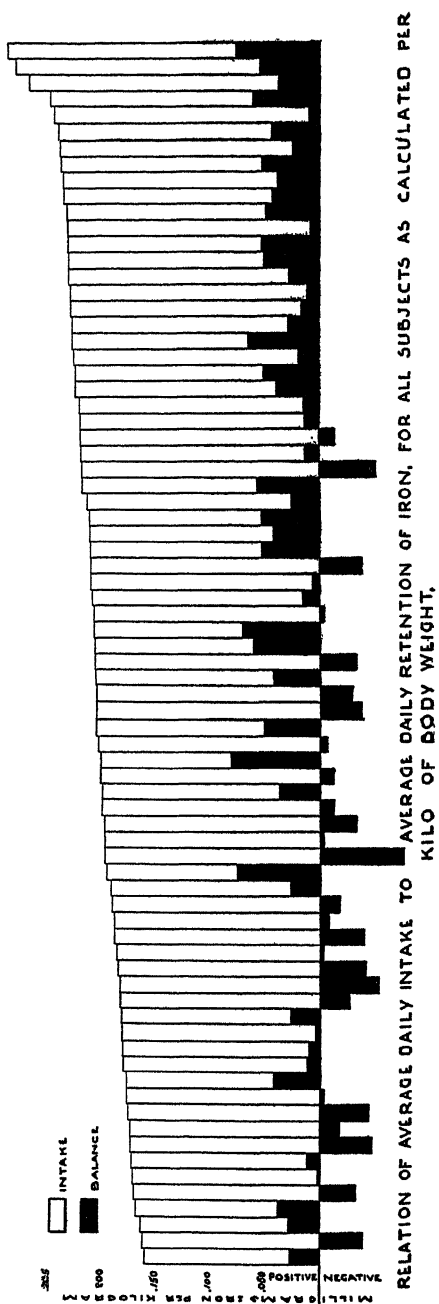
Figure 1

2.96. The average coefficients of variation are 11.9 and 25.8 for iron of food and excreta respectively. For three subjects the variability of the output is at least twice that of the intake and for the fourth subject the two are practically the same.

The variabiliy of the daily balances is best demonstrated by figure 2 which shows graphically the average daily balance of each subject for each 5-day period as given in table 2, for each 10-day period, and for each menstrual cycle. There are periods of negative and positive iron balance for each of the

TABLE 3

Statistical constants

| SUBJECT | MEAN | RANGE | | STANDARD DEVIATION | COEFFICIENT OF VARIATION |
		Lowest	Highest		
Average daily food intake					
	mg.	*mg.*	*mg.*	*mg.*	
Subject A	11.93[1]	9.86	14.16	1.20	10.1
Subject B	11.87	9.84	14.56	1.24	10.5
Subject C	10.03	7.76	13.04	1.30	13.0
Subject D	11.71	9.36	16.16	1.65	14.1
Average daily total excretion					
Subject A	12.89	7.15	25.79	4.34	33.6
Subject B	10.39	2.30	14.49	2.94	28.3
Subject C	9.32	6.85	12.13	1.30	13.9
Subject D	11.90	4.71	21.15	3.27	27.4

[1] Does not include iron from ferric ammonium citrate.

subjects during the metabolism study but no consistent regularity in the order of their occurrence can be detected. Of particular note is the independence of retention with respect to the menstrual periods. The results indicate no regularity in the way the body compensated for the menstrual losses of the subjects.

Effect of ferric ammonium citrate supplement. Beginning with the fourteenth consecutive 5-day period and continuing through the twenty-second period subject A was given a daily supplement of 5 mg. of iron in the form of ferric ammonium

AVERAGE DAILY BALANCE OF IRON
FOR EACH SUBJECT FOR EACH FIVE-DAY PERIOD,
EACH TEN-DAY PERIOD AND EACH MENSTRUAL CYCLE.
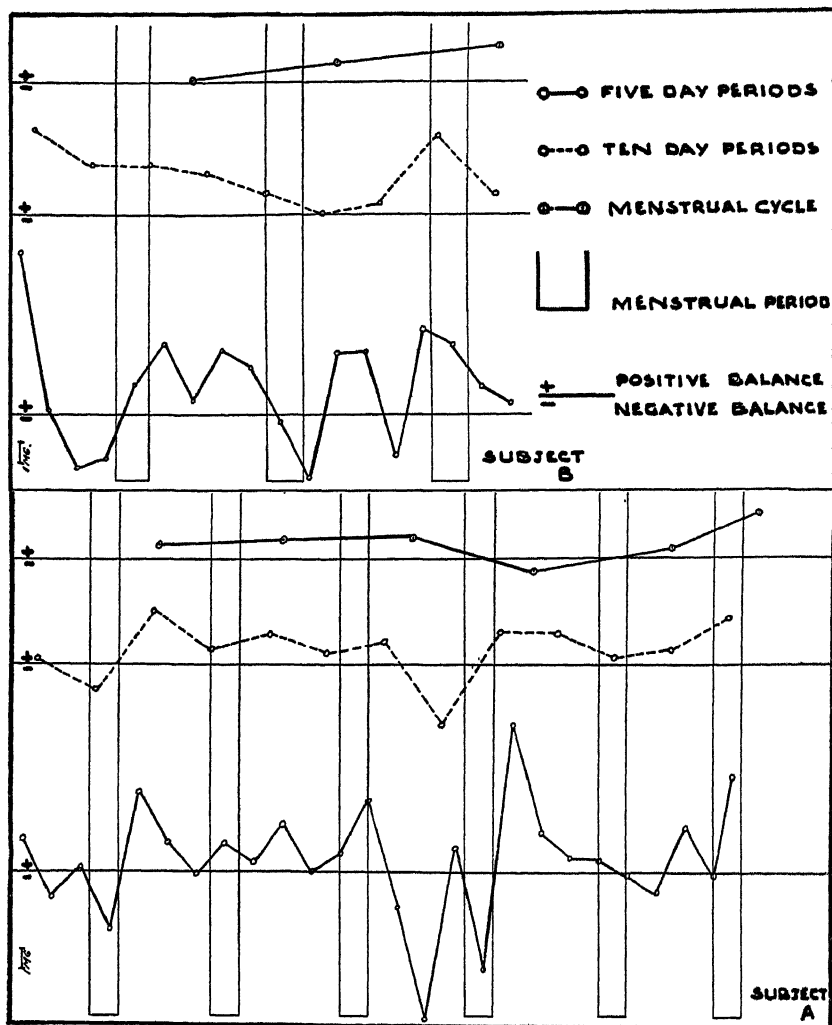
Figure 2

citrate. The effect of this is shown in table 4 where the average daily intake, excretion and balance of iron for the periods before and after the iron therapy are compared with those
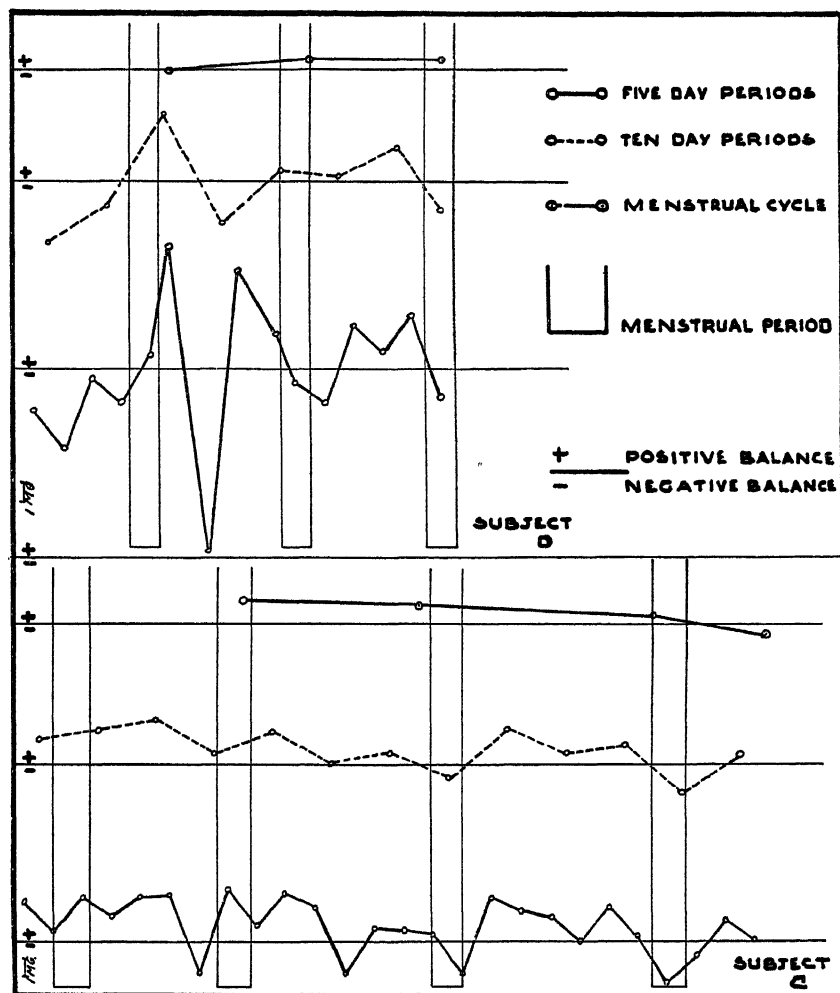
Figure 2—*Continued*

for the periods of iron therapy. During the first thirteen periods of the study subject A had an average positive balance between food and feces and urine of + 1.01 mg. of iron per

day on an average intake of 12.07, but when the average intake was increased to 16.79 by the addition of a solution of ferric ammonium citrate this balance was reduced to — 0.10 mg. per day. When this iron therapy was discontinued and the average daily intake dropped to 11.38 mg. storage took place at the average rate of 1.54 mg. per day. These results were unexpected and a possible explanation will be considered in the discussion.

Menstrual losses. A complete record of the menstrual losses of each subject during each menstrual period throughout the study is given in table 5. Using the figures for the subject's

TABLE 4

Effect of a daily supplement of ferric ammonium citrate on the iron exchange of one subject

PERIODS	TOTAL DAYS	TOTAL PER 5-DAY PERIOD			DAILY AVERAGE			PER KILOGRAM PER DAY		
		Intake	Excr.	Bal.	Intake	Excr.	Bal.	Intake	Excr.	Bal.
A-M inc.[1]	65	60.37	55.32	+5.05	12.07	11.06	+1.01	0.208	0.188	+0.020
N-V inc.[2]	45	83.95	84.47	—0.52	16.79	16.89	—0.10	0.283	0.290	—0.007
W-Z inc.[1]	20	56.90	49.20	+7.70	11.38	9.84	+1.54	0.189	0.165	+0.024

[1] No supplement.

[2] Five milligrams iron daily as ferric ammonium citrate.

own hemoglobin content in grams per 100 cc. it was possible to calculate the volume of blood represented by the iron loss for each subject for each period. Since not all the iron in the blood is in the hemoglobin molecule it is not strictly accurate to convert milligrams of iron into cubic centimeters of blood on the basis of hemoglobin content. However, for practical purposes of expression it is justified because it gives a more understandable measure of the actual menstrual loss and any discrepancies of the procedure are probably within the experimental error of the methods. Both milligrams of iron and cubic centimeters of blood are also calculated on the basis of body weight. Subjects A, B, C and D lost an average of 14.26, 22.84, 11.13 and 13.80 mg. of iron, or 30.01, 50.78, 26.48

and 29.68 cc. of blood, respectively, during each menstrual period. The most striking fact shown by the individual figures is the constant amount of iron lost in the different menstrual periods of the same subject. The variation from one period to the next is less than 10% in the periods of subjects B, C and D though greater for subject A. There appears to be no definite relationship between the iron lost in the menses and

TABLE 5

Menstrual losses of four subjects

SUBJECT	PERIOD	DURATION IN DAYS	TOTAL LOSS		LOSS PER KILOGRAM	
			Milligrams iron	Cubic centimeters blood	Milligrams iron	Cubic centimeters blood
A	I	5	10.46	23.75	0.178	0.403
	II	5	16.80	35.91	0.286	0.611
	III	6	16.40	35.05	0.279	0.597
	IV	5	11.86	23.57	0.201	0.399
	V	6	12.96	25.89	0.217	0.434
	VI	5	17.10	35.92	0.288	0.605
	Average		14.26	30.01	0.241	0.510
B	I	7	22.01	51.62	0.361	0.848
	II	7	22.37	49.73	0.369	0.821
	III	6	24.13	50.98	0.399	0.843
	Average		22.84	50.78	0.380	0.837
C	I	8	10.86	27.49	0.238	0.603
	II	7	11.26	26.98	0.245	0.587
	III	6	10.86	25.29	0.240	0.558
	IV	6	11.56	26.15	0.264	0.597
	Average		11.13	26.48	0.247	0.586
D	I	5	13.26	29.86	0.245	0.551
	II	5	14.52	31.17	0.266	0.570
	III	4	13.62	28.01	0.248	0.510
	Average		13.80	29.68	0.253	0.544

the duration of the period, or to the length of the cycle or to the body weight of the subject.

Another blood loss must be recorded for subject A who had a severe nosebleed the night preceding her fifth menstrual period. The blood was collected on cellulose pads and treated in the same way as the menses. The iron content of the blood loss was 36.57 mg. which was equivalent to about 73 cc. of

her blood. She also had had slight nosebleeds during menstrual periods II and III but because the loss was small the blood was added to the menses for those periods. After the severe loss preceding period V her nostril was cauterized and there was no recurrence of this bleeding.

TABLE 6

Balance of iron for each menstrual cycle for each subject

CYCLE	NUMBER OF DAYS	AVERAGE DAILY			BALANCE FOR CYCLE	LOSS IN MENSTRUATION	FINAL BALANCE	LOSS IN VENOUS BLOOD	ACTUAL BALANCE
		Intake	Excretion	Balance					
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Subject A									
I	22	11.22	10.91	+0.31	+6.72	10.46	—3.74	9.69	—13.43
II	21	12.66	11.68	+0.98	+20.49	16.80	+3.69	10.98	—7.29
III	23	13.96·	12.97	+0.99	+22.99	16.40	+6.59	4.68	+1.91
IV	21	17.34	17.95	—0.61	—12.69	11.86	—24.55	11.07	—35.62
V	24	15.04	14.57	+0.47	+11.44	12.96	—1.52	{ 36.57[1] 5.01	—43.10
VI	15	11.31	8.91	+2.40	+36.00	17.10	+18.90	4.76	+14.14
	Total				+84.95	—85.58	—0.63	—82.76	—83.39
Subject B									
I	29	10.79	10.64	+0.15	+4.24	22.01	—17.77	9.38	—27.15
II	27	12.57	11.48	+1.09	+29.53	22.37	+7.16	12.15	—4.99
III	28	12.52	10.55	+1.97	+55.24	24.13	+31.11	0.0	+31.11
	Total				+89.01	—68.51	+20.50	21.53	—1.03
Subject C									
I	30	10.80	9.50	+1.30	+39.06	10.86	+28.20	5.93	+22.27
II	35	10.18	9.20	+0.98	+34.49	11.26	+23.23	12.52	+10.71
III	39	10.00	9.52	+0.48	+18.85	10.86	+7.99	9.59	—1.60
IV	20	8.38	8.89	—0.51	—10.20	11.56	—21.76	6.63	—28.39
	Total				+82.20	—44.54	+37.66	34.67	+2.99
Subject D									
I	27	10.72	10.84	—0.12	—3.25	13.26	—16.51	4.44	—20.95
II	26	13.17	12.70	+0.47	+12.22	14.52	—2.30	9.32	—11.62
III	25	11.26	10.70	+0.56	+14.05	13.62	+0.43	9.72	—9.29
	Total				+23.02	—41.40	—18.38	23.48	—41.86

[1] Loss in nosebleed.

Balances for complete menstrual cycles. In order to give the most complete picture of the iron exchanges in the subjects the data are presented for each menstrual cycle, the logical unit of time for which to calculate an iron balance. In table 6

the average daily intake, excretion and balance of iron is calculated for each cycle and then the total balance for each cycle is presented together with loss in the menses and the balance between these is shown. This is designated as the 'final' balance for each subject, since it is the figure which would be of chief consideration in the usual iron metabolism of women. Since the loss in the venous blood samples and the loss by subject A in a severe nosebleed cannot be disregarded they have been subtracted from the final balance figures and the result designated as the 'actual' balance for the subjects of this study.

It may be noted from the figures given in table 6 that during thirteen of the sixteen menstrual cycles studied the average daily intake exceeded the average daily excretion. The average daily positive balances range from 0.31 mg. to 2.40 mg. with a mean value of 0.93 mg. for all subjects. During the other three cycles there were daily negative iron balances of 0.61, 0.51 and 0.12 mg. between intake and excreta.

The subjects did not always retain enough iron from their intakes to compensate for that lost in the menses. During only nine of the sixteen cycles studied was the total positive balance between intake and excretion sufficient to cover completely the iron lost in the menstrual flow and thus place the subjects in final iron equilibrium or in positive balance. In three other cycles the final negative balances were probably small enough to be negligible, 3.74, 1.52 and 2.30 mg. The final balances for the remaining four cycles were — 16.51, — 17.77, — 21.76 and — 24.55 mg.

Calculation of the actual iron balance of these subjects includes the loss of iron due to the taking of blood samples as well as that in the feces, urine and menses. In only six of the sixteen menstrual cycles studied were the subjects able to compensate for the additional iron lost from the body in the venous blood samples. When the entire time of the study is considered only subjects B and C were near actual iron equilibrium. Subject B accomplished this at one fell swoop in the last cycle by retaining 31.11 mg. which practically compensated for the 32.14 mg. lost in the two previous cycles.

Conversely subject C stored 32.98 mg. in cycles I and II and lost 29.99 mg. in cycle III and the first 20 days in cycle IV. Subject A retained enough to balance the loss in the venous blood samples only during cycles III and VI and subject D never did this. Subject A was in actual negative iron balance to the extent of 83.39 mg. and subject D of 41.86 mg. for the entire study.

Total metabolic picture for the entire study. Since much of the value of this study of iron metabolism lies in the fact that it was continuous over several months a presentation of the complete metabolic picture is important. In figure 3 the total number of milligrams of iron in the food are compared with the total loss of iron in the feces, urine and venous blood samples for each subject for the entire time of the experiment. Under ordinary conditions the final balances between the iron intake and the sum of the iron losses in the feces, urine and menses would give the complete picture of the measurable iron metabolism. Considering just these pathways of loss subjects B and C were in slight positive balance, subject A was in equilibrium, and subject D in negative balance during the entire study. However, the subjects in this study lost additional iron due to the taking of venous blood samples for calcium and phosphorus analysis. Thus when this is considered the 'actual' balances show that subjects B and C were in equilibrium and subjects A and D were in negative balance.

The intakes and losses are calculated also on the basis of milligrams of iron per kilogram per day and shown also in figure 3. By thus ruling out differences due to the varied length of time on the balance study and to unequal body weights the results for all the subjects are comparable. The sum of the losses in the feces, urine and menses for subjects A (omitting the periods of iron therapy), B, C and D were 0.201, 0.192, 0.213 and 0.219 mg. of iron per kilogram per day, respectively. The average is 0.206 mg. These figures will be used later in the calculations of minimum requirement of iron for these subjects and for other normal women.

Hemoglobin and red cell content of the blood. The results of the daily determinations of hemoglobin and red cell content have been reported in a separate article (Leverton and Roberts, '36). Analysis of the data shows the occurrence of daily variations in both hemoglobin and red cells, the majority of which are not greater than the experimental error. The standard deviation for the entire series is 0.90 for hemoglobin and 0.31 for red cells. There was a definite upward trend in the hemoglobin value of every subject during the entire period.

COMPARISON OF INTAKE AND LOSS OF IRON FOR EACH SUBJECT
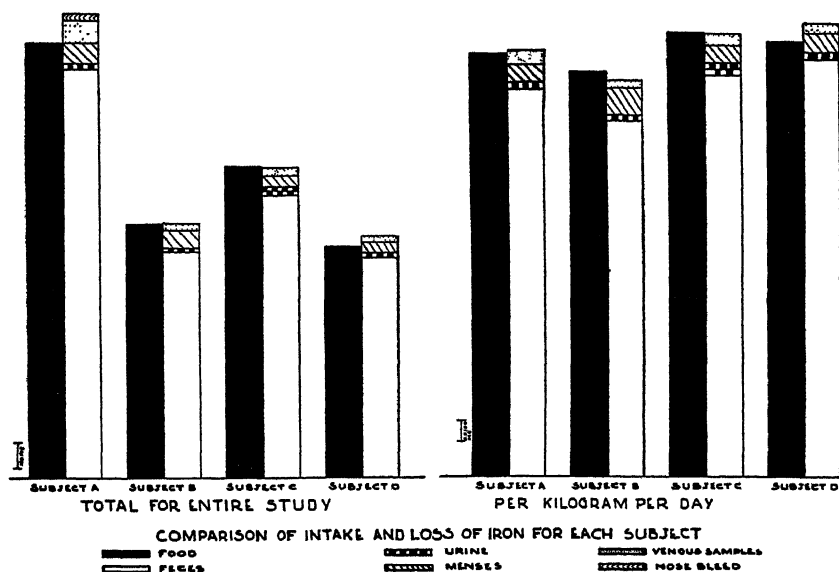
Figure 3

The values increased from 12.95, 12.54, 11.62 and 13.06 gm. per 100 cc. for subjects A, B, C and D, respectively, for the first menstrual cycle to 14.00, 13.92, 13.00 and 14.30 for the last cycle in the study. The red cell count remained remarkably constant for all subjects throughout the experiment. In subject A when 5 mg. of iron from ferric ammonium citrate was given daily during two menstrual cycles the red cell content increased 0.5 million per cubic millimeter and the hemoglobin content increased 1 gm. per 100 cc. These higher

values were not maintained when the citrate was discontinued. From the data presented it appears that there is no measurable or consistent effect of the process of menstruation upon the daily values of either hemoglobin or red cells in the subjects studied. Although occasional marked daily variations in the blood values occur they do so irrespective of the different phases of the menstrual cycle.

A comparison of the average daily iron balance of each subject with the average hemoglobin content of her blood for each 5-day period shows that no measurable relationship between these two factors exists. In many cases the hemoglobin value rises during or following the time when the body is in negative balance or vice versa and a positive balance does not necessarily parallel hemoglobin formation. That the measurement of these two values is not comparable will be emphasized in the discussion.

<div align="center">DISCUSSION</div>

The foregoing data show variations in the iron metabolism of normal young women who had only the demands of maintenance, and were under a controlled dietary regime. Because these variations occur at random during the experimental period of several months they indicate the limitations of the interpretation of results of a metabolism period which is only a few days in length.

Some of the variations observed in this study may be due to fluctuations in the iron content of the diet. Despite precautions taken to control this factor, differences in the iron content of foods due to manufacture, preservation and cooking made some variation inevitable. Also in the present study it was advisable to permit some foods ad libitum and although chosen carefully they were not iron-free and so caused some of the variation observed in the iron intakes of the subjects during the different periods. Marked fluctuation in the iron content of supposedly constant diets, and of samples of the same food taken at different times from different sources, have been reported also by Coons ('35), Toscani and Reznikoff

('34), and Davidson and LeClerc ('36). Such findings together with those of the present study indicate that analysis of each diet as eaten by each subject is the only accurate procedure for balance experiments.

Even though variations occur in the iron intakes they are not as great as the irregularities in the iron excretions and thus in the calculated balances. To what extent the rate of intestinal motility influenced the excretion of iron is speculative. All the subjects had reasonably regular habits of elimination but they did not have the same rhythm or rate of defecation. Subjects A and B had an average of three bowel movements during each 5-day period, and the carmine did not appear in their stools until the second or third morning after it was ingested. The coefficient of variation of the iron content of the excreta in these cases was 38 and 28, respectively. Subject C had two stools a day and the carmine appeared usually within 12 and always within 24 hours after it was taken. In this case the coefficient of variation was only 13, the same as for her intake. Subject D had one movement a day and the carmine always appeared the first morning after it was taken, but even with this regularity the coefficient of variation was 27. It is of interest that subjects A (omitting the periods of iron therapy) and B with a slower intestinal motility had an average daily excretion of 0.177 and 0.172 mg. of iron per kilogram for the entire study and subjects C and D with a more rapid motility had an average daily excretion of 0.204 and 0.214 per kilogram, 18% higher than for A and B. No relationship was found to exist between variations in the fiber content of the diet—entailed by the fact that apples were permitted ad libitum—and variations in the iron excretion.

Evidence of the influence of variations on the interpretation of the results of a metabolism study is demonstrated best by figure 2. In this average total daily retentions are charted for periods of different lengths—5 days, 10 days, and complete menstrual cycles. Careful study of the chart shows that the results from an isolated 5-day period or even a 10-day period would give a very different picture of the iron metabolism of these women than do the results of a continuous study

over a longer period of time. These variations in iron balances when the subjects were under reasonably controlled conditions for several months together with the fact that the variations did not lessen as the study proceeded offer striking evidence of the need for long and continuous metabolism periods if a true record of the body's activity is to be obtained.

It is of interest to compare the average menstrual losses of iron for the subjects in this study with those reported in the literature. Barer and Fowler ('36) have studied the losses of 100 normal individuals during menstruation and found an average iron loss of 19.54 mg. per period. When translated into blood losses on the basis of individual hemoglobin content the average was 50.55 cc. and the standard deviation 25.73 cc. Fifty per cent of her subjects lost between 23.21 and 68.43 cc. of blood in a period. The losses in the present study were 11, 13, 14 and 22 mg. of iron, which represented blood losses ranging from 24 to 52 cc. with an average of 34 cc. Barer's figures and those from this study show that when the amount of blood lost in the menstrual flow is calculated from actual analysis the figures are markedly lower than the usual estimates of 150 cc. to 600 cc. given in physiology textbooks.[2] This increasing knowledge that the normal menstrual losses are probably much less than were heretofore thought and therefore less alarming from the standpoint of the total quantity of blood lost is indeed acceptable. The very constant losses during different periods for the same subject in this study might suggest that in normal women on an adequate regular diet the menstrual loss is definite and characteristic for each individual. This, however, would not be concluded from the findings of Barer who reports marked variation from period to period and more variations for the subjects whose menstrual losses were greater than the average.

The results of increasing the iron intake of subject A by the addition of ferric ammonium citrate were unexpected since reports in the literature indicate that this form of iron is

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[2] It has been suggested that gynecologists are prone to disregard these figures and accept the Hoppe-Seyler figure of 37 cc. as nearer the true value.

readily available to the body. The average daily negative balance of 0.10 mg. between food and feces and urine during the periods of increased iron intake is hardly large enough to be significant, but the change in daily iron retention from + 1.01 to — 0.10 when it was added and then from — 0.10 to + 1.54 when it was withdrawn is striking. Although the astringent effect on the intestinal tract of some iron salts is known it has not been reported in the case of ferric ammonium citrate and was not expected of the 3 cc. of 0.95% solution given daily in this case.

The concurrent hemoglobin values for subject A during this time are not in accord with her condition of negative iron balance or even with a condition of equilibrium. During the cycles when she was receiving additional iron her hemoglobin increased from 13.8 to 14.8 gm. per 100 cc. of blood and then dropped to 14.0 when the supplement was withdrawn. The statistical significance of this increase has been considered in the original report (Leverton and Roberts, '36). Hemoglobin formation requires iron, but at no time during the entire study had subject A retained enough from either food or food plus supplement to account for the iron storage represented by the increase in the total hemoglobin content of her blood. This means that the hemoglobin was formed at the expense of the bodily stores of iron and that these stores were not replenished during the experimental regime. Because of the recognized role of copper in hemoglobin formation, it was suspected that the contamination of the ferric ammonium citrate by copper might be one cause for the rise in hemoglobin value. This possibility was precluded, however, when the copper content of the 5-day foundation dietary was calculated and found to be as high as 6 mg. Schlutz, Morse and Oldham ('33) and Fowler and Barer ('35) report large iron retentions and the latter coincident hemoglobin regeneration following the administration of ferric ammonium citrate. The differences between their results and the results of this supplementary iron feeding experiment may be due to several factors. Fowler and Barer used as their subjects six patients with

hypochromic anemia. Although they do not state the amount of the supplement given they report a storage as high as 6.27 gm. of iron in 24 days which would indicate a daily dose of over 250 mg. Likewise Schlutz, Morse and Oldham fed an infant a daily dose of 100 mg. of iron in this form plus 0.75 mg. of copper and found a large iron retention though practically no hemoglobin regeneration, but here again the subject was markedly anemic. Certainly these are not conditions comparable to those of subject A who was receiving only a 5 mg. supplement of iron and who was, moreover, normal when the supplement was begun.

A possible explanation of the failure of subject A to store the additional iron given her orally is found in the result of the study by Heath et al. ('32) of hemoglobin regeneration following oral and parenteral administration of ferric ammonium citrate to patients with hypochromic anemia. He found that it required a daily dose of 1000 mg. of metallic iron when given orally as ferric ammonium citrate to produce the same blood-building effect as a daily dose of 32 mg. of metallic iron when given parenterally in the same form. That absorption of the iron in this form was the limiting factor appears likely though this is not in accord with the results of the studies on the availability of iron from ferric ammonium citrate.

The lack of correlation between the iron balances and the hemoglobin values for the different periods is not unexpected when the difference in the amount of iron required to make a significant change in these values is calculated. For subject A a change of 140 mg. in the amount of iron circulating in the blood stream would be required to vary the hemoglobin value 1 gm. per 100 cc., and then because of the method used for its determination such a change would be considered significant only if it resulted from many analyses over a period of several weeks. This change nevertheless involves ten times the amount of iron lost during a single menstrual period by this subject. On the other hand a change as small as 1 mg. in the average daily total retention of iron over a shorter period of time may

be considered significant in relation to the intake or excretion. It is not to be expected that iron retention and hemoglobin formation parallel each other for the retention of iron is but one step in its utilization.

A study of the complete metabolic picture for the subjects for the entire period is important for an understanding of their actual iron requirement. Following the current practice of considering the amount of a mineral which is excreted as representing the minimum requirement for that mineral, the minimum total daily iron requirement to cover the complete normal losses during a menstrual cycle for subjects A (omitting the periods of iron therapy), B, C and D, would be 11.9, 11.7, 9.16 and 11.9 mg., respectively. It is important that these minimum intakes would not provide for the replacement of any extra or unusual loss of iron such as a nosebleed or taking venous blood samples. Even though the subjects were receiving approximately only their minimal requirement of iron from the diet they increased the hemoglobin content of their blood slightly more than 1 gm. per 100 cc. This increase represented the utilization of 120 to 140 mg. of iron which had to come from bodily stores. Further increases in hemoglobin values would have been advisable but to what extent they would have been possible without replenishment of bodily stores is a question. Furthermore it is reasonable to assume that the maintenance of a level of iron stores above that of bare minimum is in greatest accord with optimum health. This together with the fact that iron is needed in the body for purposes other than blood building indicates the need of an intake of iron for these subjects which is greater than their minimal requirement. It has been customary to add 50% to a minimal requirement of a dietary essential and designate the result as an optimal allowance. On this basis the optimal allowance for subjects A, B, C and D would be 17.8, 17.5, 14.4 and 17.8 mg. of iron daily.

These results suggest certain facts regarding the iron requirement of normal women. The average daily fecal and urinary excretion of iron for all subjects for the entire time

of the study was 0.193 mg. per kilogram. Considering this figure as the minimal requirement and 50% above this or 0.289 mg. as the optimal allowance, the respective values for a 56 kilo. woman would be 10.8 mg. and 16.2 mg. The question then arises as to whether the optimal allowance will provide for the replacement of iron lost periodically in the menses or whether a separate allowance should be made to cover this loss. This will depend on two factors, the quantity of iron lost in the menses and the length of the menstrual cycle or the time between losses when the body can compensate for the previous loss or store iron in advance of the next one. Using Barer's average figure of 19.5 mg. for the iron content of the menses and assuming a menstrual cycle of 28 days in length it may be calculated that an average daily retention of 0.7 mg. would be necessary to compensate for such a menstrual loss. The dietary standard for a normal 56 kilo. woman would thus approximate 17 mg. per day. Another method of using the results of this study to calculate the daily iron requirement of women is to take the average total loss per kilogram per day of all the subjects, increase it 50% and multiply by the weight of an average woman. Thus 0.206 mg. per kilogram per day which covers losses in the feces, urine, and menses would be increased to 0.309, and then multiplied by 56 to give the total daily optimal allowance of iron which is 17.3 mg. The results obtained by either method of calculation are higher, though in reasonable agreement with the standard of 15 mg. of iron per day recommended by Sherman ('33).

It is a well-known fact that unless particular care is taken in planning a diet it will not contain the recommended 15 mg. of iron per day. The diet in the present study bears evidence to this. It was planned to represent a well-chosen mixed dietary with no emphasis on foods rich in iron and as a result it averaged only 10 to 13 mg. per day.

Parallel with the generally accepted intakes of iron which are lower than optimum are the generally accepted lower hemoglobin values for women. The latter are usually explained as due to the periodic loss of blood in the menses and

therefore were probably unavoidable. Since our present knowledge of the relatively small losses of iron and blood in the menses minimizes this as an etiologic factor it would seem that the low hemoglobin values are more directly a reflection of the prevalence of iron-deficient diets consumed over long periods of time than of excessive losses of iron in the menses. This does not mean that even a small loss should be disregarded when it is recurrent and continues through many years as does the menstrual loss, but neither should it be used to justify low hemoglobin values if they could be corrected by the ingestion of diets optimum in iron content.

SUMMARY AND CONCLUSIONS

The total iron exchanges of four normal young women on an adequate diet during consecutive menstrual cycles were determined over a period of 3 to 5 months by means of a continuous balance experiment.

The total time of the experiment was divided arbitrarily into consecutive 5-day periods and a single foundation dietary was used for every subject during every period. Certain low-mineral foods were permitted ad libitum to meet the differing energy requirements of the subjects. Food composites which represented one-tenth the amount of all food eaten by each subject were made and analyzed for each period.

Feces and urine collections were combined for each 5-day period and analyzed for iron. The menstrual flow was collected on cellulose pads for analysis. Records were kept of any additional blood losses and the results considered in the actual iron balance of the subjects.

The average daily intakes of iron for the entire time for the four subjects were 13.61, 11.87, 10.03 and 11.71 mg. with corresponding balances between food and excreta of + 0.72, + 1.48, + 0.71 and — 0.20 mg. The average coefficient of variation was 11.9 for the iron in the food and 25.8 for that in the excreta.

Although there were periods of negative and of positive balance for the subjects during the study, a negative balance never occurred when the intake of iron from food was 0.225

mg. or more per kilogram per day. No relation between time of iron storage and its loss during the menstrual period could be demonstrated.

A supplement of 5 mg. of iron in the form of ferric ammonium citrate was given to one subject during her fourth and fifth menstrual cycles. This resulted in an increased excretion and a decreased retention of iron as compared with the cycles when she was not receiving the supplement.

The average menstrual losses for the four subjects were 14.26, 22.84, 11.13 and 13.80 mg. of iron, respectively. The losses were relatively constant from period to period for the same subject.

In nine of the sixteen menstrual cycles the subjects were in iron equilibrium or slight positive balance after the menstrual loss had been subtracted from the balance between the iron intake and iron excretion for each cycle.

Only two subjects were able to compensate also for the iron loss in the venous blood samples which were taken in connection with another study. The other two subjects were in negative iron balance when this additional loss was included in calculating their actual iron balance for the entire study.

All the subjects increased the hemoglobin content of their blood at least 1 gm. per 100 cc. during the experimental regime; the red cell content remained remarkably constant.

The findings are discussed in relation to the iron requirement of these subjects and of other normal women. These subjects on a diet which contained 10 to 14 mg. of iron daily and fortified in other dietary essentials were receiving only their minimal requirement of this element. Calculations for the optimal daily allowance of iron for a 56 kilo. woman give results of 16 to 17 mg. which is somewhat higher than the 15 mg. recommended by Sherman. The results of this study together with the fact that the average dietary seldom contains even 15 mg. of iron indicate that the low hemoglobin values which are accepted as normal for women because of the drain due to menstruation may be a direct reflection of the use of diets habitually low in iron rather than due to the small periodic loss in the menses.

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THE UTILIZATION OF ENERGY PRODUCING NUTRIMENT AND PROTEIN AS AFFECTED BY SODIUM DEFICIENCY [1]

ORME J. KAHLENBERG, ALEX BLACK AND E. B. FORBES

Institute of Animal Nutrition, Pennsylvania State College

ONE FIGURE

This study was undertaken as a unit in a series of investigations in the light of the conception that any deficiency in any essential nutrient must eventually affect food utilization in general, the specific objects of these studies being to demonstrate the ways in which, and the degrees to which individual nutritive deficiencies affect the utilization of food energy and protein.

It is natural to think of sodium, in animal nutrition, in association with chlorine, since these two elements occur in diets predominately in combination as sodium chloride; and, in regard to the functions of this salt in nutrition, the attention naturally concerns itself mainly with the chlorine, in its obviously important relation as a constituent of the gastric juice—the accompanying sodium being considered mainly in connection with its own utilization, and with the process ('alkaline tide') of its elimination from the body.

In the present study, however, chlorine and the metabolism of sodium in combination with chlorine, are eliminated from the nutritional picture, by the provision of chlorine, in abundance, to all experimental subjects, thus turning the light of inquiry upon the element sodium.

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In harmony with the extreme solubility of the salts of
sodium, and with the fact of the marine origin of terrestrial
animal life, sodium is omnipresent within the body, but is
most abundant in the extracellular fluids. It contributes to
the normal electrolyte contents of the body, and to the main-
tenance of acid-base balance, osmotic equilibrium, muscular
irritability, and control of heart action. Its functions, there-
fore, are broad and fundamental.

The literature on the functions of sodium has been reviewed
by Barker, Hoskins and Mosenthal ('22), Peters and Van
Slyke ('31), Heubner ('31), Klinke ('31), Meyer-Bisch ('31),
Sherman ('32) and Bodansky ('34).

A number of determinations have been made of the sodium
requirement of the rat. Thus, Osborne and Mendel ('18)
found that rats 'grew with vigor' on a diet containing 0.035%
of sodium, and that "less than 0.04% of either sodium or
chlorine in the food sufficed to permit these rats to complete
their growth." H. G. Miller ('23) obtained satisfactory
growth of rats on a synthetic diet containing 0.07% of sodium.
Later Miller ('26) failed to get growth from a diet containing
0.03% of sodium, but did obtain satisfactory growth when the
sodium content of the diet was increased to 0.42% sodium (our
calculation). Olson and St. John ('25) found 0.23% of sodium
in the diet of the rat to be insufficient, but found 0.53% to be
satisfactory for growth and reproduction. Mitchell and
Carman ('26) concluded, from growth and metabolism experi-
ments with rats and chickens, that "the addition of sodium
chloride to a ration composed largely of corn, containing
0.047% sodium and 0.041% chlorine, enhances its growth-
promoting value." Schoorl ('34) found that on a diet con-
taining 0.009% sodium the growth of rats was limited to the
attainment of a body weight of about 60 gm. during 10 weeks,
and that rats made a good growth on a diet with a sodium
percentage of 0.2. Richards, Godden and Husband ('24) found
that the addition of sodium chloride or citrate to a ration of
cereal grain fed to a growing pig led to increased assimilation
and retention of nitrogen, calcium and phosphorus.

Also Sjollema ('35) found the growth of chicks considerably restricted by rations poor in sodium, especially when containing no more than 0.013% of this element.

That a greater degree of concordance was not found in the foregoing observations of sodium requirements of rats was doubtless due to the facts that the influence of the accompanying chlorine intake was not in all cases excluded, and that the sodium intake and the plane of nutrition differed, as also did the judgment of the experimenters as to what constituted satisfactory growth.

No reports have come to the attention of the authors of investigations in which the relation of sodium alone to efficiency of food utilization has been studied in extensive and rigidly controlled experiments of long duration. With such considerations, therefore, the present study is concerned.

EXPERIMENTAL PROCEDURE

A sodium deficient and a sodium supplemented diet were compared by means of a 10-week growth, metabolism and body analysis study, with twelve pairs of weanling albino rats as subjects, each pair being of one sex and the same litter, with feed intake controlled by the paired method. The details of technic were the same as described in a recent paper by Swift, Kahlenberg, Voris and Forbes ('34).

The composition of the two diets was as follows:

Na-deficient diet		Na-supplemented diet	
Ground corn (maize)	78.90	Ground corn (maize)	78.90
Casein (vitamin-free)	14.00	Casein (vitamin-free)	14.00
Cod liver oil	2.00	Cod liver oil	2.00
Calcium chloride ($2H_2O$)	0.10	Calcium chloride ($2H_2O$)	0.10
Dextrin	4.00	Dextrin	1.05
Calcium carbonate	1.00	Calcium carbonate	1.00
Sodium bicarbonate	0.00	Sodium bicarbonate	1.75
Crisco	0.00	Crisco	1.20

The two diets were maintained equicaloric by adjustment of their contents of dextrin and of hydrogenated vegetable oil.[2] By analysis the supplemented diet contained 0.502%, and the deficient diet 0.007%, of sodium.

[2] Crisco.

In order to prevent an extensive storage of sodium during the preliminary growth interval the young rats and their mothers were given the sodium deficient diet from the sixteenth day after the birth of the young until the beginning of the experiment.

At the end of the experiment, the rats were killed, and the bodies were analyzed for moisture, ether extract, nitrogen and energy, as in the other experiments of this series, the heat production being computed by the body balance procedure, that is, as the difference between the gross energy of the food and the sum of the energy of the body increase and the excreta. In addition the sodium content of the rat bodies was determined.

Sodium was determined in the diets and in the rat bodies essentially by Butler and Tuthill's ('31) application of the method of Barber and Kolthoff ('28).

PRESENTATION OF RESULTS

In consideration of the complexity and the fundamental nature of the functions of sodium in nutrition it seems inevitable that the effects of sodium deficiency should be general in character; and thus they were found to be—that is, sodium deficiency was apparent especially as it affected nutrition in general, rather than in an obviously specific way.

Among the entire series of nutritive deficiency studies that have been made in this laboratory the present experiment was unique in the promptness, the decisiveness and the consistency with which the deficiency, in this case of sodium, served to diminish the rate of growth.

In this experiment there were 396 refusals of feed, 308 by the animals on the sodium deficient diet, and eighty-eight by the rats on the supplemented diet. The sodium deficient rats, therefore, restricted the food intake of their pair mates.

In relation to the quantitative results—all average data as presented represent twelve animals on continuous metabolism investigation during a 10-week period.

All the rats which received the sodium supplemented diet made greater growth than did their pair mates (fig. 1). The average gains in body weight of the rats, with contents of alimentary tract removed, on the sodium deficient and sodium supplemented diets were 31.47 gm. and 63.29 gm., respectively, the dry matter of the food eaten being 373.5 and 374.3 gm., respectively, while the dry matter of food per gram of body increase was 11.9 ± 0.26 gm. for the rats on the deficient diet, and 5.9 ± 0.22 gm. for the rats which received the sodium supplemented diet.

TABLE 1

Gain in body weight[1] of rats as related to dry matter of food

PAIR	Na-DEFICIENT DIET			Na-SUPPLEMENTED DIET		
	Food eaten (dry matter)	Gain in body weight	Dry matter of food per gram body gain	Food eaten (dry matter)	Gain in body weight	Dry matter of food per gram body gain
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	434.7	36.48	11.9	435.7	101.45	4.3
2	382.0	25.62	14.9	382.9	77.70	4.9
3	379.9	35.57	10.7	380.7	76.59	5.0
4	371.7	32.69	11.4	372.5	50.51	7.4
5	409.7	38.61	10.6	410.6	68.81	6.0
6	349.6	30.23	11.6	350.4	53.41	6.6
7	428.2	40.10	10.7	429.2	74.52	5.8
8	358.2	29.94	12.0	359.0	63.31	5.7
9	360.6	27.63	13.1	361.4	57.56	6.3
10	346.6	25.94	13.4	347.4	45.56	7.6
11	357.2	32.17	11.1	358.0	51.36	7.0
12	303.0	22.60	13.4	303.7	38.67	7.9
Average	373.5	31.47	11.9	374.3	63.29	5.9

[1] Excluding contents of alimentary tract.

Since the energy intake was the same with both groups, the greater gain of body weight by the rats on the sodium supplemented diet was necessarily made from a smaller proportionate food expenditure.

The digestibility of the diets was unaffected by the difference in sodium content, the average coefficients for nitrogen being 91.5± 0.20 and 92.4 ± 0.17%, and for energy 92.1 ± 0.08 and 92.4 ± 0.06%, respectively, for the sodium deficient and the sodium supplemented diets.
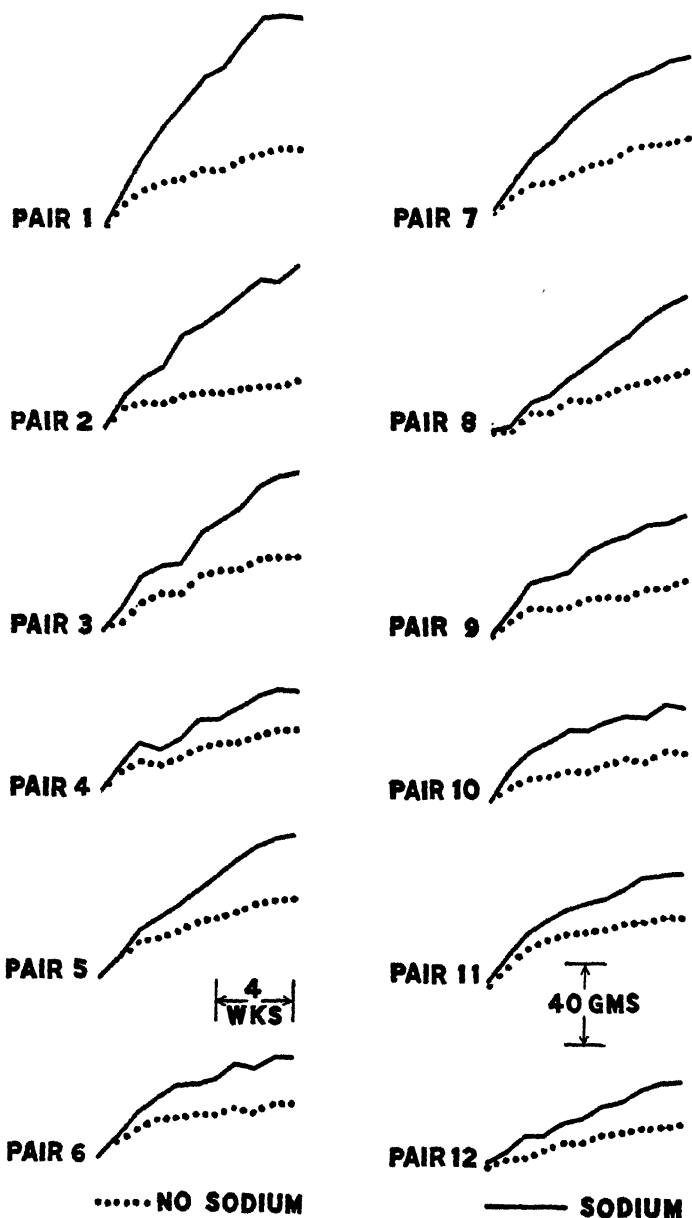
Fig. 1   The growth of albino rats, during 10 weeks, as affected by sodium deficiency.

In regard to energy storage there was a remarkable difference in favor of the sodium supplemented diet, the average body gains being 63.0 and 133.9 Calories, which were equivalent to 3.5 ± 0.15% and 7.5 ± 0.32% of the food energy, for the sodium deficient and the sodium supplemented rats, respectively.

TABLE 2

Energy of body gain of rats as related to energy and to metabolizable energy of food

PAIR	FEED ENERGY	Na-DEFICIENT DIET				Na-SUPPLEMENTED DIET			
		Body gain		Metabolizable energy	Body gain as percentage of metabolizable energy	Body gain		Metabolizable energy	Body gain as percentage of metabolizable energy
			Per cent of feed energy				Per cent of feed energy		
	Cal.	Cal.			%	Cal.			%
1	2074.9	66.5	3.2	1803.5	3.7	178.1	8.6	1830.7	9.7
2	1823.1	51.9	2.8	1601.2	3.2	167.2	9.2	1615.3	10.4
3	1813.0	64.3	3.5	1598.5	4.0	142.3	7.8	1610.5	8.8
4	1774.1	82.0	4.6	1567.8	5.2	96.6	5.4	1562.2	6.2
5	1955.2	100.4	5.1	1721.7	5.8	194.3	9.9	1728.4	11.2
6	1668.7	52.8	3.2	1475.9	3.6	121.6	7.3	1476.0	8.2
7	2043.6	82.1	4.0	1804.4	4.5	185.8	9.1	1787.2	10.4
8	1709.7	64.9	3.8	1516.2	4.3	121.0	7.1	1511.6	8.0
9	1721.2	59.1	3.4	1517.0	3.9	140.7	8.2	1522.7	9.2
10	1654.3	45.3	2.7	1469.7	3.1	72.4	4.4	1455.2	5.0
11	1704.6	53.3	3.1	1498.9	3.6	98.8	5.8	1492.9	6.6
12	1446.1	33.0	2.3	1274.3	2.6	87.5	6.1	1280.0	6.8
Aver.	1782.4	63.0	3.5	1570.8	4.0	133.9	7.5	1572.7	8.5

The metabolizable energy of the diets was essentially the same, being 1570.8 and 1572.7 Calories, respectively, the body gains of metabolizable energy being 4.0 ± 0.17 and 8.5 ± 0.36%, respectively, for the sodium deficient and the sodium supplemented diets.

The rats on the sodium supplemented diet also materially exceeded their pair mates on the deficient diet in regard to nitrogen retention. On an average the gains of nitrogen were 1.40 and 2.46 gm., which were equivalent to 10.5% and 18.5%, respectively, of the food nitrogen, for the sodium deficient and the sodium supplemented rats.

The character of the total gains in energy is indicated by the facts that of the 63.0 Calories gained by the sodium deficient rats, 47.3 Calories was in the form of protein and 15.7 Calories in the form of fat, while of the 133.9 Calories gained by the sodium supplemented rats 84.9 Calories was in the form of protein and 49.0 Calories in the form of fat.

TABLE 3

Nitrogen of body gain related to fat and energy of body gain and to nitrogen of feed

PAIR NO.	NITROGEN OF BODY GAIN	FAT GAINED		ENERGY GAINED			NITROGEN OF FEED	
			Per gram nitrogen gained	Total	As protein	As fat	.	Utilized for body gain
			Sodium deficient diet					
	gm.	*gm.*	*gm.*	*Cal.*	*Cal.*	*Cal.*	*gm.*	*%*
1	1.52	1.5	1.0	66.5	53.1	13.4	15.49	9.8
2	1.12	1.4	1.3	51.9	39.0	12.9	13.61	8.2
3	1.55	1.2	0.8	64.3	52.6	11.7	13.54	11.4
4	1.46	3.5	2.4	82.0	48.8	33.2	13.25	11.0
5	1.63	4.9	3.0	100.4	54.1	46.3	14.60	11.2
6	1.38	0.8	0.6	52.8	45.2	7.6	12.46	11.1
7	1.72	2.3	1.3	82.1	60.1	22.0	15.26	11.3
8	1.30	2.0	1.5	64.9	45.8	19.1	12.77	10.2
9	1.19	2.0	1.7	59.1	40.5	18.6	12.85	9.3
10	1.24	0.3	0.2	45.3	42.1	3.2	12.35	10.0
11	1.51	0.5	0.3	53.3	48.6	4.7	12.73	11.9
12	1.14	— 0.5	— 0.4	33.0	37.7	— 4.7	10.80	10.6
Average	1.40	1.7	1.1	63.0	47.3	15.7	13.31	10.5
			Sodium supplemented diet					
1	3.94	4.6	1.2	178.1	136.3	41.8	15.49	25.4
2	2.90	7.2	2.5	167.2	101.1	66.1	13.61	21.3
3	2.93	4.5	1.5	142.3	100.1	42.2	13.54	21.6
4	2.07	2.7	1.3	96.6	71.1	25.5	13.25	15.6
5	2.57	11.5	4.5	194.3	87.1	107.2	14.60	17.6
6	2.01	5.4	2.7	121.6	70.9	50.7	12.46	16.1
7	2.74	9.8	3.6	185.8	93.5	92.3	15.26	18.0
8	2.44	3.7	1.5	121.0	85.6	35.4	12.77	19.1
9	2.15	7.0	3.3	140.7	75.5	65.2	12.85	16.7
10	1.99	0.6	0.3	72.4	66.6	5.8	12.35	16.1
11	2.10	2.8	1.3	98.8	72.1	26.7	12.73	16.5
12	1.66	3.1	1.9	87.5	58.4	29.1	10.80	15.4
Average	2.46	5.2	2.1	133.9	84.9	49.0	13.31	18.5

The heat loss for the rats on the sodium deficient and sodium supplemented diets was 1507.8 Calories and 1438.9 Calories, or 84.6 ± 0.17 and 80.7 ± 0.31% of the food energy, respectively. The statistical significance of this difference is expressed by odds of more than 10,000 to 1.

The method of this investigation does not reveal the relative extent to which the observed difference in heat produc-

TABLE 4

Digestibility of nitrogen and energy producing nutriment

PAIR	Na-DEFICIENT DIET				Na-SUPPLEMENTED DIET			
	Nitrogen		Energy		Nitrogen		Energy	
	Feces	Digested	Feces	Digested	Feces	Digested	Feces	Digested
	gm	%	*Cal.*	%	*gm.*	%	*Cal.*	%
1	1.78	88.5	183.6	91.2	1.40	91.0	157.2	92.4
2	1.15	91.6	151.6	91.7	1.03	92.4	136.2	92.5
3	1.14	91.6	141.3	92.2	0.96	92.9	129.0	92.9
4	1.06	92.0	135.4	92.4	0.96	92.8	131.7	92.6
5	1.14	92.2	155.0	92.1	0.97	93.4	148.1	92.4
6	0.93	92.5	125.5	92.5	0.83	93.3	121.8	92.7
7	1.30	91.5	161.6	92.1	1.33	91.3	169.5	91.7
8	0.94	92.6	127.3	92.6	0.88	93.1	129.4	92.4
9	1.06	91.8	137.9	92.0	0.97	92.5	128.9	92.5
10	1.02	91.7	120.8	92.7	1.08	91.3	129.1	92.2
11	1.17	90.8	136.1	92.0	1.04	91.8	131.9	92.3
12	0.85	92.1	114.5	92.1	0.67	93.8	103.9	92.8
Average	1.13	91.5	140.9	92.1	1.01	92.4	134.7	92.4

tion is to be accounted for as maintenance quota and as energy expense of food utilization.

The markedly different sodium intake in the two diets was reflected in a moderate difference in the percentage of sodium in their bodies (0.325 ± 0.004, as compared with 0.368 ± 0.002), in favor of the rats on the sodium supplemented diet, and a decided difference, also in favor of the rats on the sodium supplemented diet, in the average quantity of sodium in their bodies (0.072 ± 0.001 gm., as compared with 0.114 ± 0.003 gm.).

The moisture content of the bodies of the rats did not differ significantly, as influenced by the diets, the average value for the sodium deficient rats being $67.6 \pm 0.28\%$, and for the sodium supplemented rats $66.9 \pm 0.35\%$.

TABLE 5

Heat loss related to energy of food

PAIR	FEED ENERGY	Na-DEFICIENT DIET		Na-SUPPLEMENTED DIET	
		Heat loss		Heat loss	
			Per cent of feed energy		Per cent of feed energy
	Cal.	*Cal.*		*Cal.*	
1	2074.9	1737.0	83.7	1652.6	79.6
2	1823.1	1549.3	85.0	1448.1	79.4
3	1813.0	1534.2	84.6	1468.2	81.0
4	1774.1	1485.8	83.7	1465.6	82.6
5	1955.2	1621.3	82.9	1534.1	78.5
6	1668.7	1423.1	85.3	1354.4	81.2
7	2043.6	1722.3	84.3	1601.4	78.4
8	1709.7	1451.3	84.9	1390.6	81.3
9	1721.2	1457.9	84.7	1382.0	80.3
10	1654.3	1424.4	86.1	1382.8	83.6
11	1704.6	1445.6	84.8	1394.1	81.8
12	1446.1	1241.3	85.8	1192.5	82.5
Average	1782.4	1507.8	84.6	1438.9	80.7

TABLE 6

The sodium content of rat bodies

PAIR NO.	SEX	SODIUM DEFICIENT DIET	SODIUM SUPPLEMENTED DIET
		gm.	*gm.*
1	M	0.070	0.157
2	M	0.073	0.129
3	M	0.081	0.129
4	M	0.076	0.109
5	F	0.074	0.115
6	F	0.068	0.100
7	F	0.080	0.122
8	M	0.068	0.108
9	F	0.064	0.100
10	F	0.069	0.098
11	M	0.076	0.101
12	F	0.065	0.094
Average		0.072	0.114

After 4 or 5 weeks on the experiment, the sodium deficient animals manifested a craving for sodium by licking and biting the fingers of the attendant, when being handled for weighing; and after 8 weeks on experiment these rats evinced an increased desire for salt by licking the sides of the cage and water bottle immediately after feeding.

At no time during the 10-week experiment, however, did the sodium deficient rats exhibit ocular or visual abnormality, or such a decline in weight as was reported by St. John ('28).

SUMMARY

In a growth, metabolism, and body analysis experiment, by the paired feeding method, sodium deficiency unfavorably affected the appetite, the increase in weight, the storage of energy and the synthesis of fat and protein—the proportion of the energy stored as fat being the larger with a sodium supplemented than with a sodium deficient diet.

There were no significant differences in the digestibility of the protein, or in the digestibility or the metabolizability of the energy producing nutrients, of the two diets, or in the moisture contents of the bodies of the two groups of rats.

The heat loss was significantly higher with the rats on the sodium deficient than with those on the sodium supplemented diet.

Decidedly more sodium was stored in the rat bodies from the sodium supplemented than from the sodium deficient diet, with resulting higher percentage of sodium in the bodies of the rats of the former group.

With food intake restricted by their pair mates on a diet containing 0.007% sodium, rats on a diet containing 0.502% sodium increased in body weight from 51 gm. to 116 gm. during 70 days, that is, at a rate of 0.9 gm. per day, while a diet containing 0.007% sodium permitted growth at only about half the rate stated.

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THE UTILIZATION OF HEXOSES BY EXCISED RAT TISSUES [1]

M. ELIZABETH MARSH

Department of Vital Economics, University of Rochester, Rochester, New York

In the preparation of artificial media for tissue metabolism studies the assumption frequently has been that glucose, in a concentration equal to or greater than that of the blood was necessary for a more normal value of the metabolism and to maintain it for any considerable period of time. Evidence has been accumulating which indicates that in certain tissues added glucose is not utilized—at least that it is not burned.

With liver tissue Crabtree ('29), Quastel and Wheatley ('33) and Kisch ('33, '34) measuring only oxygen consumption found no increase with glucose. Dickens and Greville ('33), Himwich et al. ('34) and Elliott and Baker ('35) found no change in the R.Q. as well.

With kidney tissue there are conflicting reports on the effect of glucose. Shorr, Loebel and Richardson ('30) in addition to some of the above workers, found increases in both O_2 and R.Q. when glucose was added, but Kisch found no difference in the O_2 uptake whereas Himwich obtained a rise in O_2 but only an insignificant rise in R.Q.

Richardson, Shorr and Loebel ('30) found no difference in either O_2 or R.Q. of muscle strips with and without glucose and Kisch, using rat diaphragm found no change in O_2 uptake with any of the three hexoses.

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Many of the other tissues have been found to oxidize added glucose to a considerable extent but liver and muscle seem to stand out as two which do not, although there has been but little work done with muscle. The evidence for the oxidation of glucose by kidney is conflicting.

Investigations on fructose and galactose have yielded the following results. With liver, Dickens and Greville found that fructose produced a rise in R.Q. but none in O_2 consumption, while Kisch found a slight rise in O_2 with fructose but not with galactose. The latter also found no effect of galactose on kidney but an increase with fructose, in accord with the results of Dickens and Greville.

The present research was limited to a study of the influence of the three hexoses, glucose, fructose and galactose, upon the respiratory exchange of liver, kidney and muscle tissue. In many of the groups both fed and fasted rats were employed to determine whether or not the nutritive condition of the tissues played a role in their utilization of the sugar and whether or not this circumstance might explain some of the conflicting reports in the literature.

<div align="center">METHOD</div>

The studies were carried out by means of the Warburg apparatus with a modified bottle in which measurements of both O_2 and CO_2 were made on the same tissue slices. The bottle, shown in figure 1, contains a central partition high enough to permit the bottles to be shaken with as much as 3 cc. of fluid in each half without danger of its spilling over. The tissue in Ringer's-phosphate solution is placed in one side and $N/5$ $Ba(OH)_2$ in the other side. A side arm was added to each half of the bottle to hold $N/2$ HCl. This permits the tissue and medium, as well as the alkali, to be treated with acid for the release of any CO_2 which may have been held in either. A correction is made for the amount of CO_2 in the tissue and fluid at the beginning, determined on a separate sample of tissue.

While this method still necessitates the measurement of CO_2 and O_2 in a CO_2 free atmosphere, and in a medium buffered with phosphate alone, both of which are unphysiological, it does eliminate the error which may be involved in the original Warburg method of measuring CO_2 production and O_2 absorption on different samples of tissue. Serum, however, may be used in place of the Ringer's-phosphate solution with results only slightly better for ordinary conditions of measurement at 37.5° C. (Marsh, '34).

Fig. 1   A new bottle for the Warburg apparatus, having two chambers each with side sack, permitting determination of preformed CO_2, O_2 and final CO_2.

Simultaneous determinations in triplicate were made of the oxygen consumption and of the respiratory quotient with and without the various sugars. Measurements were made for 1 hour at 37.5° C. and O_2 values are expressed in mm.^{3} per hour per milligram of dry weight of tissue. Triplicate values which varied by more than 7 to 8% were discarded. R.Q.'s seldom varied more than 0.05 and usually not more than 0.02 or 0.03. In some of the experiments on liver the determinations were made in air (as indicated in the tables) but all

others were made in oxygen. The glucose used was a pure
product supplied by Corn Industries Research Foundation
and the fructose and galactose were obtained from Pfanstiehl
Chemical Company.

The medium was the usual Ringer's solution buffered with
N/150 phosphate mixture of pH 7.4 (Warburg medium de-
scribed by Richardson, '29) and the sugar solutions were
made up in this medium to a final concentration of 0.2%. It
was found after the experiments were practically completed,
that the pH of the Ringer's-phosphate solution, as determined
by the quinhydrone method, was not 7.4 but about 7.1. How-
ever, check experiments at both reactions showed that, al-
though the O_2 values for liver were slightly higher at pH 7.4
than at 7.1, the response to the sugars was the same and with
kidney tissue there was no difference, even in the absolute
values.

The finding of Quastel and Wheatley ('34) also was con-
firmed that M/150 phosphate buffer is not strong enough to
maintain the pH of the solution in which liver is suspended.
The change may be from 0.3 to 0.6 pH in an hour. More con-
centrated solutions of phosphate precipitate calcium ions and
glycerophosphate was found to be but little better in main-
taining the original pH. With kidney tissue, there is little
or no drop in the pH of the Ringer's-phosphate solution dur-
ing an hour's determination.

RESULTS

The mean values for the oxygen consumption and the R.Q.
of kidney tissue obtained with all three sugars are sum-
marized in tables 1 and 2. Data are treated statistically with
the standard deviation, the probable error of the mean and
coefficient of variability in table 1 for both fed and fasted
rats. Table 2 gives the difference of the means, the probable
error of the difference and the ratio of these two values, which
should be over 3 if the difference between the mean values
is significant.

TABLE 1

Oxygen consumption and respiratory quotients of kidney tissues with and without sugar

MEDIUM	NUMBER OF EXPERIMENTS	O_2-MM.³/HR./MG.					B.Q.				
		Mean	Increase	S.D.	P.E. of mean	V	Mean	Theoretical R.Q.	S.D.	P.E. of mean	V
							Fed rats				
			per cent			per cent					per cent
No sugar	11	22.68		1.67	±0.24	7.4	0.767		0.032	±0.005	4.2
Glucose	11	29.41	27	1.60	±0.25	5.5	0.783	0.820	0.029	±0.004	3.7
Fructose	11	34.04	50	2.77	±0.42	8.1	0.846	0.849	0.023	±0.003	2.7
No sugar	7	24.19		2.10	±0.38	8.6	0.762		0.016	±0.003	2.1
Galactose	7	24.53	1	2.13	±0.38	8.7	0.761		0.011	±0.002	1.4
							Rats fasted 24 to 48 hours				
No sugar	10	25.86		1.40	±0.21	5.5	0.748		0.029	±0.004	3.8
Glucose	10	30.42	18	1.65	±0.24	5.4	0.746	0.786	0.022	±0.003	2.9
Fructose	10	34.66	33	3.50	±0.55	10.1	0.813	0.811	0.024	±0.004	3.0

S.D. = standard deviation. P.E. = probable error. V = coefficient of variability.

TABLE 2

Difference of the means, the P.E. of the difference of the means and the ratio of the two calculated for the data on kidney in table 1

COMPARISON OF	O_2-MM.³/HR./MG.			B.Q.		
	Difference of means	P.E. of difference of means	Difference of means / P.E. of difference	Difference of means	P.E. of difference of means	Difference of means / P.E. of difference
	Fed rats					
No sugar—glucose	6.33	±0.35	18.1	0.016	±0.006	2.6
No sugar—fructose	11.36	±0.48	23.7	0.079	±0.006	13.1
No sugar—galactose	0.34	±0.54	0.6	0.001	±0.008	0.1
	Fasted rats					
No sugar—glucose	4.56	±0.32	14.2	0.002	±0.005	0.4
No sugar—fructose	8.80	±0.59	14.9	0.065	±0.004	16.2

It will be noted that glucose produced a rise in the O_2 uptake of 27% in the kidney of fed and 18% in that of fasted animals. Such increases are significant since the ratios are 18 and 14, respectively. In contrast the R.Q. with glucose rose not at all in the fasted group and an insignificant amount in the fed group and is definitely below the theoretical R.Q. calculated on the assumption that all the extra O_2 was occasioned by the combustion of the sugar. The fact that the coefficients of variability were always lower for the R.Q. values than for the O_2 values helps to establish the accuracy of the observation that glucose does not produce a rise in R.Q. with kidney tissue in spite of a significant rise in O_2 uptake.

Fructose produced a still greater rise in O_2 consumption, viz., 50% and 33% in the fed and fasted rats, respectively, with ratios of significance of 24 and 15 in the two groups. In this case the R.Q. rose also, sufficiently to give ratios of 13 and 16 and to be within 0.002 and 0.003 of the theoretical R.Q.

Seven experiments with and without galactose showed no difference in either the O_2 uptake or the R.Q., the ratios being 0.6 for the O_2 and 0.1 for the R.Q. Only fed animals were used for this study, since there was no striking difference in the response between fed and fasted animals to the other two sugars and the little difference observed being in favor of the fed animal.

Attention might be called to the absolute values of the O_2 consumption of kidney slices measured in oxygen without sugar. A mean value of 22.7 mm.[3] for one series and of 24.2 for another shorter series is considerably higher than many of the values reported in the literature, including a previous one from this laboratory (Marsh, '34). In our own work, thinner slices were responsible for the higher values and slices too thick to permit adequate diffusion of O_2 may afford the explanation for other low values. Since the Q_{O_2} of the kidney is two to three times that of liver and most of the other tissues, the slices should be correspondingly thinner.

The results on liver are summarized in tables 3 and 4. In several of the groups, values obtained in air are averaged with those obtained in oxygen since the R.Q. and the response to the sugars were the same in both. Such mixed groups do not lend themselves to statistical treatment, however, because

TABLE 3

Oxygen consumption and respiratory quotient of liver tissue with and without sugars

MEDIUM	NUMBER OF EXPERI-MENTS	O_2-MM.^{3}/HR./MG.			R.Q.		
		Mean	Difference	Difference	Mean	Difference	Theoretical R.Q.
Fed rats							
				per cent			
No sugar	17[1]	8.56			0.721		
Glucose		8.26	—0.30	—4.6	0.720	—0.001	
No sugar	9[2]	8.19			0.722		
Fructose		8.82	+0.63	+7.7	0.814	+0.092	0.741
Glucose	14[3]	5.27			0.737		
Fructose		5.80	+0.53	+10.1	0.819	+0.082	0.760
Galactose	11[4]	7.18			0.727		
Fructose		8.30	+1.12	+15.6	0.830	+0.103	0.764
Fasted rats							
No sugar	10[5]	6.36			0.593		
Glucose		6.55	+0.19	+3.0	0.595	+0.002	
No sugar	10[6]	6.22			0.597		
Fructose		7.35	+1.13	+18.0	0.726	+0.129	0.659
Glucose	5[7]	4.86			0.597		
Fructose		5.45	+0.59	+12.1	0.725	+0.128	0.630
Galactose	11[8]	5.16			0.601		
Fructose		5.51	+0.35	+6.8	0.735	+0.134	0.626

[1] Three in air. [2] Two in air. [3] All in air. [4] Four in air.
[5] Five in air. [6] Five in air. [7] All in air. [8] All in air.

the absolute values for the oxygen absorption were definitely lower in air than in oxygen. This would mean a high standard deviation and a correspondingly low ratio of significance, even in groups in which the percentage increase with sugar is undoubtedly significant (15 and 19% in two instances). Table 4 gives the probable errors, the standard deviations and

significance ratios for only those groups in which the experiments were either all done in air or all done in oxygen.

With neither the fed nor fasted liver did glucose produce any effect. There was no rise in the oxygen uptake or in the R.Q. Oxygen absorption with fructose, when compared to that obtained without sugar, with glucose and with galactose was, in each case, higher (table 3). A larger number of experiments would undoubtedly make the percentage increase

TABLE 4

Standard deviation, probable error and ratio of significance of difference of the means to the probable error of the difference calculated for parts of the data on liver given in table 3

MEDIUM	O_2-MM.^{3}/HR./MG.			R.Q.		
	Mean	S.D.	Diff. of means / P.E. of diff.	Mean	S.D.	Diff. of means / P.E. of diff.
Fed rats						
No sugar[1]	8.84±0.11	0.42	0.58 ── = 3.2 ±0.18	0.731±0.008	0.030	0.085 ──── = 5.6 ±0.015
Fructose	9.42±0.15	0.60		0.816±0.013	0.051	
Glucose	5.27±0.08	0.47	0.53 ── = 4.8 ±0.11	0.737±0.008	0.047	0.78 ──── = 7.8 ±0.010
Fructose	5.80±0.08	0.47		0.819±0.006	0.032	
Fasted rats						
Glucose	4.86±0.10	0.32	0.59 ── = 4.5 ±0.13	0.597±0.008	0.032	0.128 ──── = 12.8 ±0.010
Fructose	5.45±0.08	0.27		0.725±0.006	0.022	
Galactose	5.16±0.10	0.15	0.35 ── = 2.3 ±0.15	0.601±0.009	0.048	0.134 ──── = 12.2 ±0.011
Fructose	5.51±0.12	0.58		0.735±0.007	0.037	

[1] Seven of the nine experiments listed in table 3, which were done in O_2. All other groups were done in air.

more uniform, since it is evident, particularly from a consideration of the R.Q.'s that liver burned neither the glucose nor the galactose.

There is no consistent difference between the fed and fasted groups, the average rise in O_2 with fructose being 11% in the fed series and 12% in the fasted.

For the groups treated statistically, the ratio of the difference of the means to the probable error of the difference is, for the O_2 absorption, under 5 in each case. Since a ratio of 3

is not a very rigid requirement for measuring the significant difference of the two groups of observations, the rise in O_2 with fructose might be questioned. However, the fact that, in fifty-four out of sixty experiments on both fed and fasted animals, there was a higher O_2 uptake with fructose, makes it seem quite certain that the sugar does produce a definite though small rise in O_2 consumption.

With liver, fructose always caused the R.Q. to rise considerably above the theoretical value for the increase in O_2 uptake, from 0.06 to 0.07 higher in the fed and from 0.07 to 0.11 in the fasted series. The ratio of significance is fairly high and higher in the fasted series.

The low R.Q.'s of liver are of interest and are very consistent in all the groups. The average without sugar is from 0.72 to 0.74 for the fed animals and from 0.59 to 0.60 for the fasted.

Diaphragm was chosen as representative of skeletal muscle since the whole muscle can be used, little injury is involved and it respires at a very uniform rate for several hours. Young rats were used to make certain that the diaphragm was thin enough to allow adequate O_2 diffusion. Two types of experiment were employed. The usual one, involving three bottles without sugar and three with, did not give entirely satisfactory results since with whole diaphragms from different rats, even though litter mates were chosen, the triplicate determinations in many instances, varied over rather a wide range. However, thirty-two experiments of this type (table 5) averaged 5.48 and 5.60 for the O_2 uptake with and without glucose and 0.871 and 0.840 for the R.Q., the rise in the R.Q. being within the error of the method. With fructose the thirteen experiments of this type are too few, considering the wide variation in the triplicate determinations, from which to draw any conclusion, but the number is sufficient to show that there was no trend toward an increase in either the O_2 or the R.Q.

However, the second type of experiment, which proved more satisfactory, gave results on O_2 uptake in accordance with

those obtainable by the usual method. This consisted of placing a solution of the sugars in the side arm above the tissue and measuring the O_2 uptake for an hour (readings every 20 minutes) in Ringer's-phosphate solution, then pouring in the sugar and following the respiration another hour. Obviously it was not possible to make R.Q. determinations under the two conditions with this method, but the mean O_2 values for fifteen such experiments with glucose and twenty-one with fructose showed, in agreement with the findings by the first method, that there was no acceleration of the O_2 absorption with either glucose or fructose.

TABLE 5

Effect of glucose and fructose on oxygen consumption and R.Q. of muscle. Diaphragm of young fed rats

MEDIUM	NUMBER OF EXPERIMENTS	O_2-MM.^{3}/HR./MG.		R.Q.	
		Mean	Difference	Mean	Difference
			per cent		
No sugar	32	5.60		0.840	
Glucose		5.48	—2.1	0.871	+0.031
No sugar	13	6.64		0.897	
Fructose		6.56	—1.2	0.890	—0.007

DISCUSSION

The data presented in this paper, together with that found in the literature, furnish definite proof of the striking differences in the ability of the tissues to oxidize monosaccharides in vitro. One would expect that the sugar of the blood would be easily and universally oxidized. Such is not the case. Of the three tissues studied, only kidney has its respiratory metabolism altered by glucose in the surrounding medium. In these experiments, in agreement with those of Himwich et al. ('34) the effect seems to be more in the nature of a stimulation of the metabolism than a direct oxidation of the sugar since it was only the O_2 absorption which was increased. Other observers (Shorr, Loebel and Richardson, '30; Dickens and Greville, '33; and Ellicott and Baker, '35) found evidence of a direct oxidation with a rise in R.Q. as well as in O_2 up-

take. The reason for this difference between our results and theirs is not apparent.

With liver and muscle, two tissues actively concerned with carbohydrate metabolism in vivo, no increase in combustion with the addition of glucose can be obtained by the present methods of studying these tissues in vitro. This is a constant finding of many observers in the case of the liver, and of the few who have worked on muscle. The latter also does not utilize fructose and these results stand in opposition to those obtained by perfusion experiments. Steinberg ('27) e.g., found that all three tissues, muscle, liver and kidney, utilized both glucose and fructose during perfusion.

There is general agreement that fructose is oxidized directly and easily by kidney. With liver, fructose produces a definite change in the metabolism characterized by a small rise in O_2 uptake and a very considerable rise in CO_2 output. Dickens and Greville, in only three and four experiments with and without fructose failed to find any rise in O_2 although the CO_2 increased.

The lack of response of liver and kidney (also muscle according to Kisch, '33) to galactose is not so surprising from a consideration of the difficulty that the rat experiences with this sugar. Harding, Grant and Glaister ('34) found, 1 hour after feeding rats galactose, 114 mg. per cent unchanged in the liver and 33 mg. per cent in the muscles.

The R.Q. of muscle, as usually found, is higher than that of kidney and liver (between 0.85 and 0.90) and indicates a high but not exclusive carbohydrate metabolism. It is not possible to explain the indifference of excised muscle to added sugars from the experiments at hand.

A consideration of the available data on the metabolism of liver in vitro greatly tempts one to speculate concerning the processes involved. Definite answers, however, are not forthcoming at present. Does the excessive increase in the CO_2 production with fructose mean that the liver burns only a small part of the sugar and converts another small part directly to fat? Excess CO_2 rather than diminished O_2 is to be expected from carbohydrate to fat conversion (Bleibtreu, '01) and

fructose is known to form glycogen more readily than glucose (Cori, '26; Feyder and Pierce, '35). The high R.Q.'s obtained in the intact body after fructose feedings are generally ascribed to a greater fat formation with fructose than with glucose (Deuel, '36).

The liver of rats fasted 24 to 48 hours gives an average R.Q. as low as Gemmill and Holmes ('35) found after 2 to 9 days of fat feeding. Do these low quotients indicate, as those authors suggest that liver, in contrast with other tissues, has the ability to convert fat to carbohydrate? Cori and Shine ('35) believe that the newly formed carbohydrate of liver found by Gemmill came from glycerol but this assumption leaves unexplained an average R.Q. of 0.58. If all the glycerol were converted to glucose, the R.Q. would not be lower than 0.68. At all events, if the low R.Q. is to be interpreted as evidence of conversion, the process must occur in fasting rats as well as in those fed on fat for several days.

In the intact animal are these two processes, carbohydrate to fat and fat to carbohydrate, going on regularly with the possibility of one or the other predominating at any particular time when the animal is killed and the liver removed? Could this be the explanation of R.Q.'s found in the literature for liver ranging from 0.45 to well over 1.0? Such a wide range of values is found for no other tissue.

SUMMARY

With a modified Warburg, measurements were made on kidney and liver slices and on whole diaphragm muscle of oxygen absorption and of respiratory quotient in Ringer's-phosphate solutions and in solutions of Ringer's phosphate to which had been added glucose, fructose or galactose. With kidney and liver both fed rats and animals fasted 24 to 48 hours were used.

Galactose was without influence on the respiration of kidney tissue, glucose increased the O_2 uptake without changing the R.Q. and fructose caused a greater increase in O_2 than did glucose, together with a rise in R.Q. which corresponded exactly to the theoretical rise.

Muscle tissue oxidized neither glucose nor fructose and liver tissue was intermediate between the two. It oxidized neither glucose nor galactose but fructose it did burn, to some extent, as indicated by a small increase in the O_2 consumption and a rise in R.Q. The latter, however, was so much higher than the theoretical rise as to suggest that processes other than oxidation were taking place, e.g., the conversion of some of the fructose to fat.

The possible role of the liver in carbohydrate metabolism is discussed.

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A COMPARISON OF THE GLYCINE CONTENTS OF THE PROTEINS OF NORMAL AND CHONDRO-DYSTROPHIC CHICK EMBRYOS AT DIFFERENT STAGES OF DEVELOPMENT

A. R. PATTON

Department of Poultry Husbandry, Cornell University, Ithaca [1]

Patton and Palmer ('36) recently reported that glycine is synthesized during the development of the hen's egg, and that a significant difference is found between the glycine contents of the proteins of normal and chondrodystrophic chick embryos. The present paper is an extension and a substantiation of their work.

Glycine has been determined in sixty normal embryos, and in eighty-three chondrodystrophic embryos. The latter were obtained by Hutt and his associates in the routine examination of about 7000 embryos which died during incubation. The method of Patton ('35) was used, excepting that calculations were made on the basis of dry weight, each sample of four eggs being acetone-alcohol-ether extracted (48 hours each) and dried 24 hours at 100°C.

Table 1 presents evidence of glycine synthesis during development, in both White Leghorn and Barred Plymouth Rock embryos. Triplicate analyses were made. The percentages for Barred Plymouth Rock embryos, from 12 to 21 days, were obtained by adding the separate values for yolks and embryos in table 2. Curves plotted from these data show minimal

TABLE 1

Glycine content of normal embryos, entire egg

AGE	GLYCINE			
	Sample 1	Sample 2	Sample 3	Mean
White Leghorn				
days	%	%	%	%
3	1.35	1.32	1.29	1.32
6	1.27	1.27	1.27	1.27
9	1.28	1.25	1.22	1.25
12	1.27	1.27	1.27	1.27
15	1.61	1.45	1.53	1.53
18	2.05	1.92	1.88	1.95
21	2.08	1.87	1.92	1.96
Barred Plymouth Rock				
0	1.76	1.73		1.75
3	1.76	1.68	1.73	1.72
6	1.08	0.52	0.60	0.73
9	1.48	0.92	1.40	1.27
12	Summation of yolk and embryo			1.10
15	Summation of yolk and embryo			1.56
18	Summation of yolk and embryo			1.80
21	Summation of yolk and embryo			2.20

TABLE 2

Glycine content of normal Barred Plymouth Rock embryos and yolks

AGE	DRY WEIGHT	GLYCINE			
		Sample 1	Sample 2	Sample 3	Mean
Embryos only					
days	*gm.*	%	%	%	%
12	1.4	1.88	1.81	1.84	1.84
15	6.3	2.06	2.20		2.13
18	10.4	2.14	2.28	2.20	2.21
21	16.7	2.37	2.28	2.28	2.31
Yolks only					
12	20.6	1.00	1.00		1.00
15	17.0	1.00	1.08		1.04
18	11.5	0.84	0.84		0.84
21	4.8	0.92	0.90		0.91

points at about 9 days, suggesting the possibility of a downward curve of glycine destruction at the beginning of incubation, encountering in normal embryos an upward curve of glycine synthesis, which becomes pronounced at 12 days—approximately the age of highest mortality from chondrodystrophy.

If glycine in embryos and corresponding yolks is determined separately (table 2), and if the relative weight changes are

TABLE 3

Glycine content of chondrodystrophic embryos without yolks

BREED	AGE AT DEATH	EMBRYOS	GLYCINE			
			Sample 1	Sample 2	Sample 3	Mean
	days	*no.*	%	%	%	%
B.P.R.[1]	10–14	10	1.40	1.56	1.40	1.45
B.P.R.	10–14	5	1.24	1.24	1.32	1.27
B.P.R.	14	3	1.16	1.00	1.00	1.05
B.P.R.	15	3	1.40	1.24	1.24	1.29
B.P.R.	20	1	1.40	1.24	1.40	1.35
B.P.R.	21	1	1.56	1.48	1.40	1.48
B.P.R.	15	3				0.88
B.P.R.	19	1				0.76
B.P.R.	19	1				1.15
Wh.L.[2]	10	21				0.78
Wh.L.	11	12				0.88
Wh.L.	12	9				1.00
Wh.L.	18	2				1.57
Wh.L.	18	1				0.95
Wh.L.	21	1				1.64

[1] Barred Plymouth Rock.
[2] White Leghorn.

taken into account, the glycine synthesis is even more evident than in the whole egg.

The percentages of glycine found in chondrodystrophic embryos (table 3) fall far below those for normal embryos of comparable ages (tables 1 and 2).

It seems evident from these data that glycine is synthesized during development, and that chondrodystrophic embryos contain less than normal amounts of glycine. The development of cartilage, bone and down is greatly retarded in

chondrodystrophy. These tissues contain proteins high in glycine content. Whether the lack of glycine in chondrodystrophic chicks is a cause, or a result, of chondrodystrophy cannot be established by the present technique.

SUMMARY

1. Glycine is synthesized during the development of White Leghorn and Barred Plymouth Rock chick embryos.

2. Chicks which die of chondrodystrophy during development contain less than normal amounts of glycine.

3. The nature of the relation between chondrodystrophy and the low value for glycine has not been established.

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IRON RETENTION IN INFANCY [1]

GENEVIEVE STEARNS AND DOROTHY STINGER

Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City

The iron content of the body at birth as determined by direct analysis (Camerer and Söldner, '00) averages about 350 mg. On the basis of existing data for tissue weights and iron content of those tissues richest in iron (Scammon, '23; Thoenes and Aschaffenburg, '34; White House Conference Reports, '32) it appears probable that the iron content of the body at 6 months may be no larger than at birth, while during the second half-year, it increases about 200 mg. It has been estimated that, during the first 2 months of life when hemoglobin normally is being destroyed, the average artificially fed infant excretes approximately 75 mg. more iron than he ingests (Stearns and McKinley, '37). To provide the iron necessary during the remainder of the first year of life, the infant would need to retain 0.75 to 1.0 mg. of iron daily after 2 months of age. The purpose of this study has been to determine the quantities of iron actually retained by infants from 7 to 54 weeks of age who are given various types of feeding.

Since the bulk of the infant's diet consists of milk, which is poor in iron, it has become customary to add to the diet some readily digestible food which is relatively rich in iron. Egg yolk is perhaps the most commonly used of these additional foods; cereals, vegetable and fruit purees are also given.

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Egg yolk, pureed spinach, and a special iron-rich cereal[2] were used for food sources of iron in this study.

The iron content of milk has been variously estimated as from 0.014 to 0.24 mg. per 100 cc. (table 1). A part of this variation is undoubtedly due to methods of analysis, a part to methods of handling the milk. These factors are probably greater than the differences among individual samples of milk.

The iron content of the additional foods used is also variable (Wallgren, '31–'32; Peterson and Elvehjem, '28). In this laboratory, analyses of egg yolks showed an average of 4.4 mg. of iron per 100 gm. Since yolks vary in weight from 12 to 22 gm., the addition of an egg yolk to the day's diet may be expected to increase the iron intake from 0.5 to 1.0 mg. daily. The pureed spinach used contained 1.22 mg. of iron per 100 gm., the cereal, 30 mg. per 100 gm.

The effect of other minerals in the diet upon the quantity of iron retained is a factor of importance because of the high mineral intake of infants. von Wendt ('05) observed that the addition of calcium sulfate to the diet of an adult man increased his iron retention. Shelling and Josephs ('34) found that in rats, an increased calcium intake decreased the retention of iron. These latter results were confirmed by Kletzien ('35) who reported also that increased intakes of sodium or potassium resulted in increased retention of iron by rats. No direct experiments have been found concerning the effect of the intake of other salts upon the retention of iron by infants. It has often been observed, however, that, although the iron content of human milk is also low, much more iron is retained by infants fed human milk than by those fed cow's milk. In addition to its lower phosphorus content, human milk contains more potassium and less calcium than cow's milk; potassium salts are often added to infants foods, in order to render the salt relationships more comparable to those of human milk. The effect upon the iron retention of the addition of potassium carbonate or chloride was therefore studied.

[2] Pablum.

TABLE 1
Iron content of cow's milk

AUTHOR	DATE	METHOD OF HANDLING MILK	METHOD USED	IRON
				mg. per 100 cc.
Sherman	1907[1]	Commercial samples		0.24
Edelstein-Csonka	1911	Drawn directly into glass containers		0.05
Peterson-Elvehjem	1928		Ferric thiocyanate	0.24
Telfer	1930			Range: 0.07–0.11 Average 0.08
Cunningham	1931	Drawn directly into glass containers		0.065
Davies	1931	Fresh Milk Pasteurized Sterilized	Ferric thiocyanate	Range: 0.15–0.24 0.17–0.38 0.16–0.28
Stugart	1931	Certified commercial samples	Modification of ferric thiocyanate method	0.044 0.046 0.053 0.073
Reis and Chakmakjian	1932	Drawn directly into glass containers	Prussian blue	0.14–0.15
Wallgren	1932	Drawn directly into glass containers	Iodimetric titration	Range: 0.014–0.032 Average 0.024
Rowett Research Institute [2]				Range: 0.07–0.11 Average 0.08
This laboratory	1935	Commercial samples	Stugart's modification of ferric thiocyanate method	0.072 0.060 0.038

[1] Published in U. S. Dept. Agric. Bull., vol. 185, 1907; same value used in Sherman, Chemistry of Food and Nutrition, 1932.

[2] Quoted from Davidson and Leitch, Nutr. Abstr. and Rev., vol. 3, 1934.

Fourteen healthy infants served as subjects. Their hemo-globin values were determined at intervals during the study and averaged 11.9 gm. per 100 cc. One infant was given human milk; the basic milk formula for the others consisted of evaporated milk containing a cod liver oil concentrate. The milk feeding was acidified with citric acid, and contained 6% of added carbohydrate, a dry dextrin-maltose mixture. Distilled water was used for making up the feedings. Each infant was given 2 ounces of orange juice daily. The egg yolk, potassium and iron salts, when fed, were added to the formula, spinach and cereal were fed separately. Potassium chloride was given in amounts of 1.8 gm., and potassium carbonate, 1.67 gm. daily, approximately doubling the day's intake of potassium. From 1.25 to 5 cc. of a 1% solution of ferric ammonium citrate was added to the day's feeding when the retention of iron from a soluble iron salt was studied. The oldest infant (D.L.) after the age of 48 weeks, received other vegetables and fruits as well as those men-tioned above. During the experimental periods, the day's feeding for each infant was quantitatively prepared and an aliquot saved for analysis. All refusals were carefully measured and subtracted from the daily intake. The infant was given the diet to be studied for several days prior to the experimental period, which was always of 3 days duration. Many short periods of study, rather than a few longer periods, were chosen because the necessary restriction of motion of the infant during the experimental periods is tiring, and it was felt that the error introduced by excessive fatigue of the infant would be greater than the analytical error intro-duced by the shortness of the period. The latter error is minimized by the number of periods studied. The copper intake was not determined. As none of the dietary con-stituents was especially purified, the copper content was assumed to be ample.

Diets and feces were digested with 20% iron-free HCl. Aliquot portions of these digests and of urine were ashed in a muffle furnace at just below red heat, the ash dissolved

in iron-free HCl and the iron determined according to the method of Stugart ('31). At the beginning of the study, porcelain crucibles were used for the ashing process, but it was observed that, as the glaze became attacked, a small iron blank was obtained from the crucible, particularly in those which had been used for ashing urine. Whenever sufficient material was available, the analyses were repeated, using platinum crucibles. The differences observed were very small, and did not affect materially the quantity of retention obtained; therefore all of the results are reported, even though for the studies of infants C.P., R.S. and E.T., porcelain crucibles were used in ashing some of the samples.

The results of the experiments are listed for each baby in table 2, and summarized graphically according to age in figure 1, and according to iron intake in figure 2.

When the milk formula was the sole source of iron, the day's intake varied from 0.66 to 2.06 mg., averaging 1.14 mg., or 0.19 mg. per kilogram. The range of retention was wide, from − 0.80 to 0.74 mg. a day, with an average daily loss of 0.05 mg., or slightly less than 0.01 mg. per kilogram. With this diet no consistent relationship could be observed between the intake level and the amount of iron retained (fig. 2) nor did the retention seem to vary in relation to the age of the infant (fig. 1).

When the diet was supplemented with egg yolk, the average intake was increased to 1.83 mg. daily (0.28 mg. per kilogram); the average retention was not increased. Pureed spinach was then given as a source of additional iron. Considerable difficulty was encountered in feeding the spinach and the average intake of iron was increased only to 1.25 mg. daily or 0.19 mg. per kilogram. The average iron loss was greater, − 0.11 mg. daily, than when the milk formula alone was given. Schlutz, Morse and Oldham ('33) fed infants dried, pureed and raw spinach and were able to increase the iron intake from 60 to 170%, but observed no significant increase in iron retention.

TABLE 2

Intake and retention of iron by normal infants given various sources of dietary iron

INFANT	AGE	WEIGHT	DIET[1]	INTAKE PER KILOGRAM	RETENTION PER KILOGRAM
	weeks	kg.		mg.	mg.
R.F.	7	5.3	B	0.17	+ 0.02
	8	5.5	B	0.20	0.00
	12	6.1	B	0.22	+ 0.08
	15	6.3	BFe	0.57	+ 0.04
	17	6.9	BFe	0.58	− 0.17
	18	7.1	BFeK	0.55	+ 0.25
	20	7.3	BFeK	0.32	− 0.20
	21	7.3	BE	0.27	+ 0.01
	23	7.7	BE	0.24	+ 0.04
	24	7.6	BS	0.12	− 0.05
	25	7.7	BFe	0.42	+ 0.11
	28	7.8	BS	0.17	− 0.05
	30	8.1	BE	0.31	− 0.06
	36	8.7	BC	0.56	+ 0.15
R.Sc	8	4.2	B	0.17	− 0.03
	10	4.6	B	0.18	− 0.01
	12	4.8	B	0.18	− 0.10
	14	5.0	BK	0.14	− 0.04
	16	5.1	BFe	0.20	+ 0.01
	17	5.2	BFe	4.20	+ 0.88
	21	5.3	BFe	1.1	+ 0.23
	22	5.4	BKFe	0.79	+ 0.10
	23	5.7	BKFe	1.25	+ 0.43
	27	6.1	BE	0.45	− 0.06
	28	6.2	BE	0.45	− 0.02
	34	6.6	BE	0.28	− 0.04

INFANT	AGE	WEIGHT	DIET[1]	INTAKE PER KILOGRAM	RETENTION PER KILOGRAM
	weeks	kg.		mg.	mg.
E.H.	16	5.1	B	0.23	+ 0.03
	18	5.5	B	0.29	+ 0.13
	22	5.9	BS	0.19	− 0.04
	24	6.4	BE	0.25	+ 0.07
	25	6.5	BFe	0.42	+ 0.02
	26	6.5	B	0.15	+ 0.05
	27	6.7	BES	0.30	+ 0.07
R.B.	17	5.9	B	0.25	− 0.14
	18	6.1	BS	0.35	+ 0.08
	20	6.0	B	0.17	− 0.06
	24	6.8	BE	0.25	+ 0.01
	25	6.9	BS	0.18	− 0.01
	26	6.5	BS	0.23	+ 0.03
	27	7.0	BE	0.26	+ 0.01
C.P.	18	6.3	BFe	1.54	+ 0.07
	19	6.5	BFe	1.17	+ 0.51
	20	6.8	BFe	1.38	+ 0.20
	22	7.7	BFeK	1.30	− 0.66
	23	7.9	BFeK	1.38	+ 0.39
	26	8.6	BFeK	0.94	− 0.63
D.L.	18	5.6	B	0.18	− 0.04
	20	5.9	B	0.21	− 0.04
	21	5.9	BS	0.17	− 0.06
	22	6.1	BE	0.19	− 0.04

	No.	Wt.	Diet	Fe	Balance	Name	No.	Wt.	Diet	Fe	Balance
D.G.	7	5.3	B	0.28	+ 0.10		26	6.1	BFe	0.69	+ 0.29
	9	5.7	B	0.16	− 0.01		27	6.2	B	0.14	+ 0.01
	11	6.1	BFe	0.50	+ 0.04		29	6.9	BES	0.40	0.0
	12	6.3	BE	0.26	− 0.08		36	7.7	BEC	0.47	+ 0.06
	13	6.4	BS	0.12	− 0.04		37	7.8	BEC	0.49	+ 0.17
	15	6.8	BE	0.22	− 0.04		48	8.5	BE	0.32	− 0.11
	16	6.8	BES	0.25	− 0.01		49	8.4	BE	0.32	+ 0.09
							53	8.4	BEC	0.81	+ 0.16
							54	8.6	BEC	0.79	+ 0.09
M.M.	10	5.0	B	0.32	+ 0.04	R.Sw.	23	6.6	BFe	1.36	+ 0.68
	12	5.4	B	0.12	− 0.04		24	6.6	BFe	1.50	+ 0.28
	14	5.7	BFe	0.91	+ 0.32		27	7.2	BFeK	1.34	+ 0.16
	16	6.1	BS	0.20	− 0.01		28	7.2	BFeK	1.34	+ 0.20
	17	6.2	BE	0.25	− 0.07		31	7.6	BFeK	1.38	+ 0.03
	19	6.4	BE	0.24	− 0.01						
	21	6.9	BE	0.18	+ 0.12						
C.B.	11	5.8	B	0.18	− 0.01	E.T.	25	6.6	B	0.21	− 0.02
	13	6.1	B	0.16	− 0.01		26	6.9	B	0.19	− 0.04
	15	6.4	B	0.11	− 0.04		28	7.3	B	0.20	+ 0.01
	16	6.5	BFe	0.40	− 0.02		29	7.3	B	0.16	+ 0.03
	21	7.2	B	0.11	− 0.07		31	7.7	BK	0.21	− 0.04
	28	8.5	BC	0.40	+ 0.09		32	7.8	BK	0.26	+ 0.01
R.M.	12	5.8	B	0.24	− 0.06	G.S.	9	5.8	HM	0.06	0.0
	13	6.2	BFe	0.29	− 0.07		13	5.7	HM	0.08	0.0
	15	6.3	BFe	0.42	− 0.12		15	6.9	HM	0.08	+ 0.03
	25	7.6	BFe	0.58	+ 0.12		18	7.2	HME	0.17	+ 0.06
							21	7.4	HME	0.15	+ 0.06
							23	7.8	HME	0.15	+ 0.02
R.N.	13	6.5	B	0.20	0.0		24	8.0	HME	0.14	+ 0.04
	14	6.6	BFe	0.43	− 0.22		25	8.2	HMBE	0.12	+ 0.05
	16	6.9	BFe	0.39	− 0.46		26	8.5	BE	0.25	+ 0.02
	28	8.4	BFe	0.61	+ 0.01						

¹ B = basal diet; K = potassium chloride or carbonate; Fe = ferric ammonium citrate; E = egg; S = spinach; C = special cereal mixture; HM = human milk.
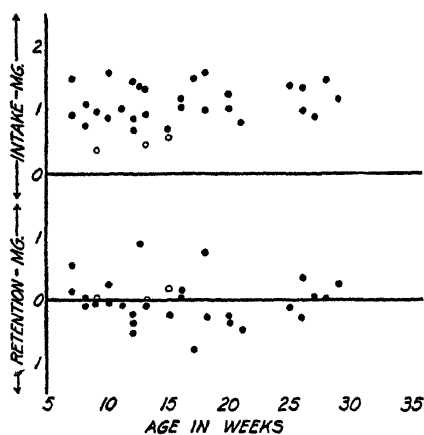
Fig. 1   Daily intakes and retentions of iron of infants from 5 to 30 weeks of age.   The dots represent studies of infants given evaporated milk feedings; the circles, studies of an infant given human milk.
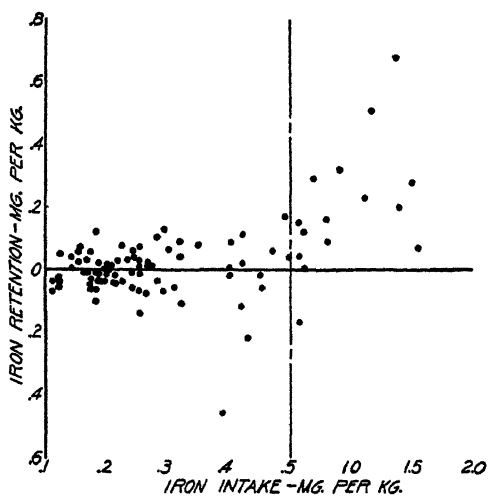

Fig. 2   The relation of iron retention to intake.   Data plotted as milligrams of iron per kilogram of body weight daily.

The quantity of spinach given to the infants of the present study, while insufficient to increase the iron intake materially, did affect the retention of calcium by the infants. During the thirteen periods when spinach was given, the calcium retention averaged 27% of the intake whereas during the twenty-six periods of study preceding and following the spinach feeding, these same infants retained 35% of the calcium intake. It is admitted that the spinach feeding was not continued for more than 10 days at a time, so the findings are not representative of a diet to which the baby has become fully adjusted. It is doubtful whether spinach would be a constant part of any infant's diet, so probably few infants would handle it better than these. In general, therefore, the feeding of spinach to infants seems more detrimental than beneficial, at least in so far as calcium and iron are concerned.

During three periods of study, both egg yolk and spinach were added to the diet. The intake of iron averaged 2.14 mg. daily or 0.32 mg. per kilogram, the average daily retention was 0.13 mg. or 0.02 mg. per kilogram, a positive though very small retention.

In view of the failure of these food sources of iron to increase sufficiently the amounts retained by the infants, the question arose as to whether infants of these ages could retain iron added to the diet in any form. A solution of ferric ammonium citrate was then added to the feedings in amounts sufficient to give daily iron intakes of from 2.6 to 21.8 mg., or from 0.29 to 4.20 mg. per kilogram. Of the seven periods of study wherein the iron intake was below 0.5 mg. per kilogram, only two showed a positive retention. The average intake of this group was 0.39 mg. per kilogram daily, the average retention − 0.17 mg. per kilogram, a poorer retention than that observed from the diets alone. When the iron intake was increased to from 0.5 to 1.0 mg. per kilogram daily, only one negative balance was found, the average retention for the six periods of study being 0.09 mg. per kilogram, with an average intake of 0.64 mg. per kilogram. A further increase in intake to from 1.0 to 1.6 mg. per kilogram

resulted in very good retentions. The intake averaged 1.34 mg. per kilogram, for the six periods studied, and the retention averaged 0.33 mg. per kilogram, or approximately 24% of the intake. It thus appears that the chief factor in determining the quantity of iron retained is the quantity ingested and that intakes of iron below 0.5 mg. per kilogram a day are insufficient to permit constant positive retentions by the average infant fed cow's milk.

To test this theory with iron of the diet from food sources, a special cereal mixture unusually rich in food iron was given in amounts of from 7 to 15 gm. daily. Egg yolk was also added to some of the diets. The daily iron intakes were adjusted to approximate 0.5 mg. per kilogram, and varied from 0.40 to 0.81 mg. per kilogram, or from 3.41 to 6.81 mg. for the total daily ingestion. Positive retentions of iron were obtained in all six periods of study, averaging 20% of the intake. When the intake was between 0.4 and 0.5 mg. per kilogram, the average retention was 0.10 mg. per kilogram, in contrast to the negative balance of − 0.17 mg. per kilogram observed when ferric citrate was the chief source of added iron. With intakes above 0.5 mg. per kilogram, averaging 0.72 mg. per kilogram, the retentions varied from 0.09 to 0.16 mg. per kilogram with an average retention of 0.13 mg. per kilogram. It seems from these experiments that iron from food sources, when given in sufficient amounts, is at least as well handled as is iron fed as ferric salt.

In figure 2, the intakes of iron per kilogram body weight have been plotted against the retention per kilogram. The data include the periods of study with the basal diet, and the basal diet plus egg, spinach, cereal, or iron salts. The chart indicates that, regardless of the source of iron in the diet of the artificially fed infants, the average retention is negligible when the daily intake is less than 0.5 mg. per kilogram. The failure of egg or spinach feeding in increasing the iron retention can be explained wholly by the fact that neither increased the iron intake sufficiently. From the retentions observed in these studies, an intake of from 1.0 to 1.5 mg.

of iron per kilogram body weight would seem ample to permit sufficient retentions of iron. These amounts are much lower than the intake advised by Elvehjem and his co-workers ('35). These authors found that maximum increases in hemoglobin level were obtained when 25 mg. of iron and 1 mg. of copper were given daily, and that increases in hemoglobin were not so consistently observed when the intakes were below this level.

RETENTION OF IRON BY AN INFANT FED HUMAN MILK

One infant (G.S.) was given human milk for three periods of study, then human milk supplemented with egg yolk, with egg yolk and cow's milk, and finally was given the same basal diet as the other infants studied, together with an egg yolk daily. The daily intake of iron increased from 0.45 mg. during the first period to 1.14 mg., when supplemented with egg yolk, and to 2.11 mg. for the cow's milk-egg yolk diet. The baby neither lost nor retained iron during the first two periods, but retained some iron during each succeeding period studied. The iron retained when human milk only was fed, averaged 0.08 mg. daily or slightly more than 0.01 mg. per kilogram The addition of egg yolk increased the daily retention to 0.32 mg. (0.045 mg. per kilogram). When the milk of the diet was supplied by equal portions of cow's and human milk, given as separate feedings, the iron intake was approximately the same, 1.03 mg., and the daily retention 0.39 mg., a retention quite comparable to that when human milk and egg alone were fed. During the one period when no human milk was given, although the daily intake of iron was doubled, 2.11 mg., the daily retention dropped to 0.20 mg. Wallgren ('33) found that three of five infants given human milk retained satisfactory quantities of iron. Other investigators (Krasnogorsky, '06; Telfer, '30) have observed, in general, much greater retention of iron from human than from cow's milk, and Maurer and his collaborators ('34) observed that substitutions of human milk for a part of the cow's milk of the feeding increased the iron retention of the infant.

THE EFFECT OF INTAKE OF PHOSPHORUS, CALCIUM AND POTASSIUM UPON IRON RETENTION

It seemed logical that the high phosphate content of cow's milk, relative to its iron content, might be the factor responsible for the poorer absorption of iron from cow's than from human milk. The iron retention per kilogram daily was therefore plotted against the intake ratio of phosphorus to iron (fig. 3). The dots represent iron intakes of 0.5 mg. per kilogram or above; the circles, iron intakes below 0.5 mg. per kilogram and the triangles, the periods when human milk
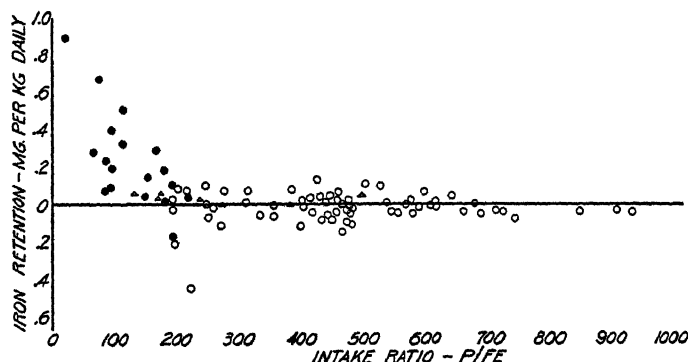
Fig. 3   The relationship retention of iron and the phosphorus: iron intake ratio. Dots represent intakes of iron greater than 0.5 mg. per kilogram daily; the circles, iron intakes less than 0.5 mg. per kilogram daily; the triangles, iron retention from human milk.

was fed. The values obtained indicate that the phosphorus intake could be increased from 200 to 1000 times the iron intake without effect upon the iron retention. The increasing iron retentions as the phosphorus: iron intake ratio decreased below 200, can be ascribed solely to the increase in iron intake. The iron retentions from human milk are no greater than many of the retentions observed when the babies were fed cow's milk.

The iron retentions per kilogram daily compared with the calcium: iron intake ratios (fig. 4), show a curve similar to that obtained with the phosphorus: iron values. Consistent

increases in iron retention were always observed when the iron intake was increased to more than 0.5 mg. per kilogram, but increasing the calcium intake from 200 to 1200 times the iron intake was without effect upon the iron retention, regardless of whether the source of iron and calcium was human or cow's milk. These findings accord with neither those of von Wendt ('05) nor Kletzien ('35).

To study the possible effect of the potassium intake upon the iron retention, potassium chloride or carbonate was added to the feeding in amounts sufficient to double the potassium
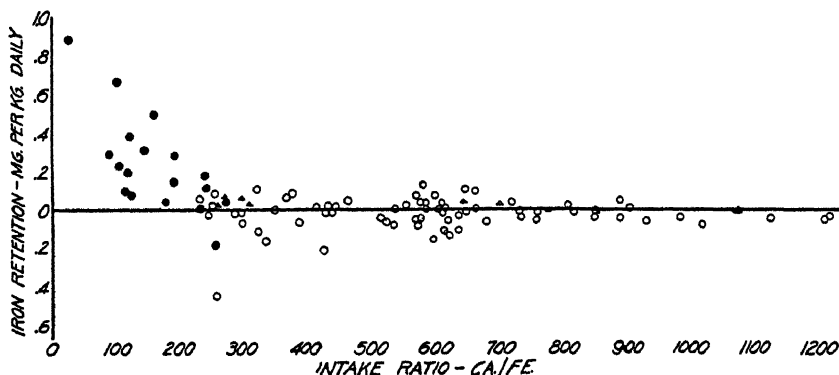
Fig. 4   Relationship between retention of iron and calcium: iron intake ratios. Symbols as in figure 3.

intake. Ferric ammonium citrate was also added. The average intake of iron was 0.86 mg. per kilogram, the average retention 0.00. This very poor average retention was due chiefly to two periods of study of baby C.P. When potassium salts were first added to his diet the result was a heavy loss of iron from the body; after the salt had been in the diet for about 2 weeks, the baby again retained iron. After a week without the salt addition, when KCl was replaced in the diet, the retention again became strongly negative. Omitting the periods of study of this baby, the average intake of iron was 0.76 mg. per kilogram, the retention 0.09 mg. per kilogram or 12%. Of eleven control periods studied, using the same infants given iron but not potassium salts, the average intake

was 0.91 mg. per kilogram, the average retention 0.18 mg. per kilogram or 20%. The age range of the infants was the same. These results do not confirm those of Kletzien ('35) upon rats, and indicate that the addition of potassium salts to the infant dietary is of no benefit to and may have a deleterious effect upon the iron retention.

SUMMARY

The retention of iron by fourteen infants varying from 7 to 54 weeks of age has been studied throughout a total of 107 3-day balance periods. One infant was fed human milk, the others were given a basal diet of cow's milk, carbohydrate and orange juice.

The daily iron intake was increased by giving egg yolk, spinach, a special cereal, or an iron salt, and the effect upon the retentions observed. The influence of the potassium, calcium and phosphorus intake levels upon the retention of iron has also been studied.

The baby given human milk was never in negative balance, although the retention was always small. Those given cow's milk feedings alone, lost an average of 0.05 mg. of iron daily. The age of the infant had no apparent influence upon the ability to retain iron. Neither egg yolk nor spinach, in the amounts given, increased the iron retention. The retention was definitely increased when the infants were given the special iron-rich cereal or ferric ammonium citrate.

No consistent relationship was observed between the iron retention and the intake of potassium, calcium or phosphorus.

From studies of the iron retention after the ingestion of varying amounts of iron as food or as soluble salt, it appears that an intake of approximately 0.5 mg. per kilogram body weight is necessary to insure a retention of iron, and an intake of 1 to 1.5 mg. per kilogram permits ample retention. Ample retentions were observed with these intakes of iron, whether the source of iron was from food (special cereal and egg) or from a ferric salt (ferric ammonium citrate).

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THE CONSERVATION OF BLOOD IRON DURING THE PERIOD OF PHYSIOLOGICAL HEMOGLOBIN DESTRUCTION IN EARLY INFANCY [1]

GENEVIEVE STEARNS AND JOHN B. McKINLEY

Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City

ONE FIGURE

The amount of hemoglobin in the blood at birth is higher than at any time subsequently and averages 22 to 23 gm. per 100 cc.[2] A rapid decrease occurs during the first weeks of life. Wide individual differences are observed in the duration of the period of hemoglobin destruction, but by the tenth week of life the amount of hemoglobin has usually decreased to 11 to 13 gm. per 100 cc., at which value it remains with slight fluctuations during the next 2 years. The purpose of this report is to record the results of an investigation concerning the fate of the iron freed by the breakdown of hemoglobin during the early weeks of life.

Studies of many years ago showed that the human fetus accumulates some store of iron in the liver and spleen. Because of the small size of the spleen, its total storage is almost negligible. The liver weighs approximately 135 gm. at birth (Scammon, '23) and, according to some observations, contains 0.026% of non-hemoglobin iron (Thoenes and Aschaffenburg, '34) or a total of 35 mg. Other data indicate an average total storage of 40 to 60 mg. (Gladstone, '32; Toverud, '35).

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[2] Throughout this discusson, whenever several sources are quoted, references are omitted. The major articles are listed in the reference list.

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Strauss ('33) has shown recently that the liver storage is inconstant and may be negligible if the diet of the mother has been low in iron. Thus the amount of iron in the liver at birth may vary from a negligible quantity to a total of possibly 60 mg. At 6 months of age the average weight of the liver is 240 gm. and its stored iron has decreased to 0.006%, or a total of 15 mg. (Scammon, '23; Thoenes and Aschaffenburg, '34). During the first 6 months of life the blood volume increases 200 cc. or more. On the basis of the average value of 12 gm. of hemoglobin per 100 cc. of blood and the content of 3.35 mg. of iron in each gram of hemoglobin (Butterfield, '09), the 200 cc. increase in blood represents 80 mg. of iron. This amount alone, exclusive of the iron needed for muscle growth, is considerably greater than the difference between the amount of iron stored at birth and that present at 6 months of age, or greater even than the total storage at birth. Obviously the iron stored in the liver at birth cannot represent the major source of iron for the formation of new blood and tissue.

The postnatal decrease of 10 to 12 gm. of hemoglobin per 100 cc. of blood in a baby whose total blood volume approximates 450 cc., liberates 150 to 180 mg. of iron, an amount sufficient for the formation of 300 to 450 cc. of new blood if the iron can all be stored for subsequent utilization. Thus the amount of iron freed by the physiological destruction of hemoglobin in early infancy exceeds greatly the amount in storage at the time of birth and obviously is the infant's largest potential source of iron. It is therefore desirable to ascertain what proportion of the freed iron is salvaged by the infant.

The evidence in the literature concerning iron conservation in early infancy is incomplete. Gladstone ('32) made both chemical and histological studies of the iron content of human fetuses and infants from the fourth month of intrauterine to the third month of postnatal life and found the maximum iron content of the liver was reached after birth, at from 1 to 10 weeks of age. This finding constitutes evidence that some storage occurred of the iron released during the period of

physiological blood destruction. Evidence that not all the released iron is stored is found in the studies of Beck ('32) who observed that iron excretion increased from the second day after birth and reached a maximum between the thirtieth and the fortieth day, then gradually decreased. Other investigators have found fecal iron values of young infants fed human milk varying from 0.35 to 2.57 mg. daily. Langstein and Edelstein ('13) studied a breast-fed newborn infant and found in consecutive 5-day studies, losses of 0.65, 1.28, 0.04 and 0.12 mg. daily, whereas two infants, 8 and 12 weeks of age, retained 0.18 and 0.26 mg. daily, respectively. On the other hand, Lichtenstein ('21) studied four breast-fed infants from 3 to 19 weeks of age and found no positive retentions in any of the twelve periods of study. The losses varied from 0.02 to 0.48 mg. daily, with an average loss of 0.25 mg. per day. The quantity of loss bore no consistent relation to age. Stearns and Stinger ('37) found that infants from 7 to 29 weeks of age, given feedings of cow's milk mixtures, showed variable retentions and losses but averaged the slight loss of 0.05 mg. daily. Again no relation was observed between age and iron retention or loss.

The evidence at hand thus indicates that during the early weeks of life the excretion of iron almost invariably exceeds the intake, but that after the second month the iron balance, though variable, tends to approach zero.

As the total amount of iron which disappears from the blood varies with the individual infant, it is necessary to follow both the level of blood iron and the daily excretion and intake in order to determine the quantity of iron freed from hemoglobin but not excreted from the body. In this study determinations of blood iron were carried out at short intervals and iron excretion was observed for several periods of varying length each during the first 2 months of life.

The infants were brought under observation as soon after birth as possible, usually at 10 days of age. The excreta were collected on pads of cellucotton. The collection for the entire period was digested with 10% iron-free HCl until a homogeneous suspension resulted. The mixture was cooled, diluted

to a convenient volume, thoroughly mixed and aliquots taken for analysis. Iron was determined by Stugart's method ('31), correcting for the iron content of the cellucotton and reagents used. Urinalyses were not made, as the iron content of urine of young infants is negligible. The iron content of each of the separate foods used, evaporated milk, a dry dextrin-maltose mixture, orange juice, and distilled water, was determined and the daily iron intake of each infant calculated.

For the blood studies, venous blood was taken from the longitudinal sinus, oxalated and well mixed. Hemoglobin was determined by the Newcomer method ('23) using a standard calibrated color disc. Cell volume was obtained by the use of the Van Allen hematocrit ('25); standard pipettes and hemocytometers were employed in making cell counts. Fowweather's method ('26) for iron content of blood was followed except for the substitution of amyl alcohol for acetone in extracting the ferric thiocyanate from the dilute aqueous solution. Standard iron solutions for both blood and fecal iron determinations were prepared from pure iron wire.

The iron excretion of seven infants was studied during the period of blood destruction. As it was deemed undesirable to take blood samples too frequently from the same infant, a larger group was used for the blood study. An average of seven analyses for each week of age was obtained from the seventeen infants studied in this series.

<div align="center">BLOOD VALUES</div>

Table 1 contains a summary of the data from determinations of the cell volume, number of erythrocytes per cubic millimeter, hemoglobin and iron content of the blood of these infants. The individual values for each determination are shown graphically in figure 1, the average values at birth being taken from the literature, and the average curves from the values of table 1.

It will be observed from the chart that, while each of the plotted components is at its highest level at birth and reaches

TABLE 1

Summary of blood data

AGE	NUMBER OF INFANTS	AVERAGE WEIGHT	CELL VOLUME	RED BLOOD CELLS	HEMOGLOBIN			CALCULATED BLOOD IRON FROM HEMOGLOBIN[1]	DETERMINED BLOOD IRON	ESTIMATED TOTAL BLOOD IRON[2]
					Minimum	Maximum	Average			
days		gm.	%	millions per cu. mm.	gm. per 100 cc.			mg. per 100 cc.	mg. per 100 cc.	mg.
10–14	5	3426	36.8	4.45	14.55	21.04	17.18	57.6	58.4	216
14–21	10	3590	34.9	4.44	12.40	20.14	15.58	52.2	51.7	194
21–28	10	3793	30.8	4.16	12.05	16.91	14.48	48.5	46.1	195
28–35	12	4040	27.0	3.92	10.51	15.08	12.41	41.6	39.2	168
35–42	8	4235	25.3	3.77	10.28	13.96	11.70	39.3	37.3	172
42–49	10	4465	25.9	3.91	9.23	15.08	11.82	39.6	39.2	189
49–56	7	4485	23.9	4.05	9.79	12.11	10.52	35.2	35.2	185
56–63	10	5051	25.9	3.49	10.20	12.05	10.82	36.3	34.0	184
63–70	6	4876	26.8	3.54	9.71	12.51	10.69	35.7	32.8	176
70–77	5	5614	26.1	3.40	10.52	11.48	11.15	37.3	33.2	224
77–84	8	5638	25.8	3.72	10.44	12.05	10.99	36.9	34.7	215
84–91	6	5583	25.4	3.52	10.11	11.71	11.11	37.2	33.8	211
91–98	8	5717	26.3	3.66	9.02	11.48	10.58	35.4	32.7	206
98–105	2	5588	25.0	4.06					33.3	204
105–112	5	5367	29.0	3.51	10.11	12.28	10.98	36.8	33.1	194
112–119	4	6165	29.1	3.74	11.14	11.38	11.27	37.7	34.4	232
119–126	6	6105	30.6	4.14	11.00	12.05	11.58	38.8	35.2	235
126–140	5	6031	28.6	4.25	11.05	12.17	11.52	38.6	34.3	226
140–194	8	6211	29.5	3.70	11.10	13.19	11.80	39.5	33.1	225

[1] The blood volume up to the fourteenth day was taken as 14.7% of body weight, and after the fourteenth day as 10.9% of body weight (Lucas and Dearing, '21). The Butterfield ('09) factor, 3.35 mg. of iron per gram of hemoglobin, was used in converting hemoglobin to iron.

a minimum at approximately the same time, less parallelism is observed between the cell volume, erythrocyte count and hemoglobin than perhaps might be expected. The hematopoietic mechanism of the infant is most unstable; the blood may be flooded with immature red cells, and its water content may vary from day to day (Abt, '35). In fact, Drucker ('24)
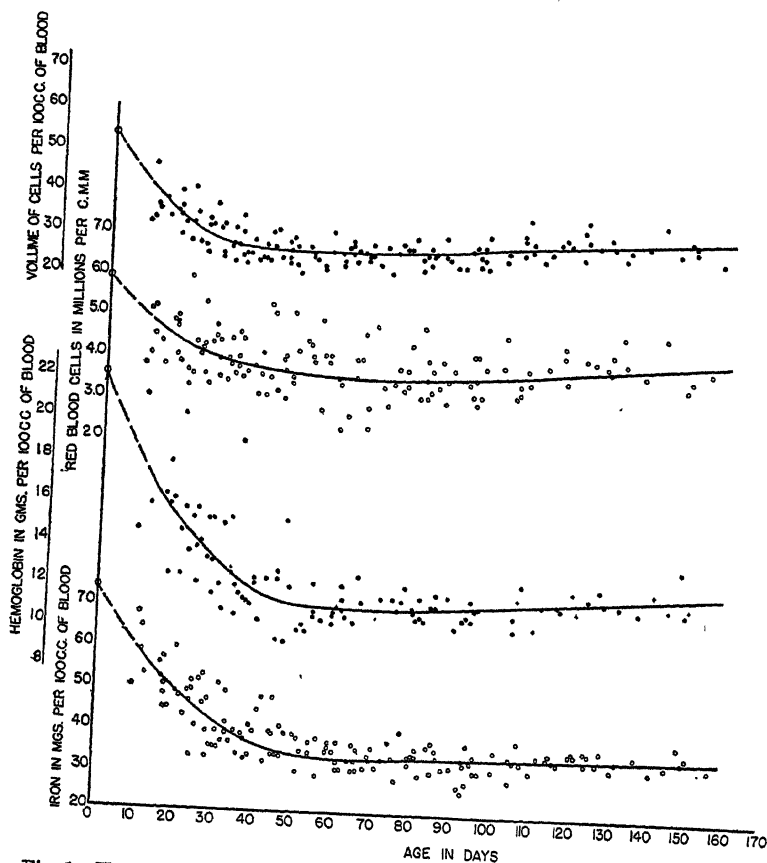

Fig. 1 Values obtained from repeated analyses of blood of seventeen infants between 10 and 160 days of age. The average values at birth are estimated; the curves are drawn from the average values obtained at the different ages. Cell volumes and hemoglobin are shown in per cent, red cells in millions per cubic centimeter of blood; and iron in milligrams per 100 cc. Sinus blood was used, except for a few hemoglobin determinations noted by +, which were determined on capillary blood.

has commented that the range of hemoglobin in the first 2 weeks of life is too great for the determination to have any clinical significance. Notwithstanding the variability, certain definite trends are observed.

The cell volume, which was estimated as 54% at birth (Mugrage and Andresen, '36), decreased to 24% by the seventh to the eighth week and then increased with minor fluctuations, to 30% at about the twentieth week. Mugrage and Andresen ('36) reported a minimal cell volume of 34% occurring between the eighth to the sixteenth week, with an increase to 37% by the thirty-second week.

The erythrocyte count at birth was estimated from the data in the literature as 5.85 millions per cubic millimeter. The values obtained are exceedingly variable during the first weeks of life and the number of erythrocytes is not as consistent with the cell volume as is observed in older infants and children. The average red cell count had decreased to 3.92 millions per cubic millimeter by the sixth week and the minimal value of 3.4 millions per cubic millimeter was reached by the eleventh week of age. The number of erythrocytes then increased to about 4 millions per cubic millimeter. Other average minimal values reported vary from 3.10 millions per cubic millimeter at 10 weeks (McLean and Caffey, '25) to 4.61 millions per cubic millimeter at 15 weeks (Merritt and Davidson, '33).

The hemoglobin at birth averages about 22 gm. per 100 cc. At the beginning of the study when the infants were 10 to 14 days of age, the average hemoglobin was 17.2 gm. per 100 cc., a value lower than the 22.2 gm. per 100 cc. reported by Elvehjem et al. ('33) and the 18.9 gm. reported by Appleton ('18). At 2 to 3 weeks the average hemoglobin was 15.6 gm., approximately equal to that observed by Mackay ('33) and higher than the average of 14.1 gm. observed by Appleton ('18). Other investigators, however, have found from 16 to 18 gm. of hemoglobin per 100 cc. in infants from 2 to 4 weeks of age. The lowest hemoglobin level, 10.5 gm. per 100 cc., was observed during the seventh and the eighth

week of life. The minimal hemoglobin level is usually reported to occur between the fifth and the twelfth week, the values as determined by different authors varying from 9.6 to 12.6 gm. per 100 cc.

The hemoglobin iron was calculated by using 3.35 mg. as the amount of iron per gram of hemoglobin (Butterfield, '09). Until about the fourteenth day of life the determined blood iron for each infant was greater than the calculated hemoglobin iron, the average values being 58.4 and 57.6 mg. per 100 cc., respectively. After this period of rapid fall the calculated hemoglobin iron was consistently greater than the determined iron. The minimal iron value of 32.8 mg. per 100 cc. was reached at 9 to 10 weeks of age, about 2 weeks later than the low level for hemoglobin. During the latter part of the study the values approached 35 mg. of iron per 100 cc. of blood.

In comparison with values of many other workers the hematologic values in this study tend to be low. The use of oxalated venous blood instead of capillary blood as is customary, could not have been the major cause. According to Lucas and Dearing ('21), hemoglobin is higher in sinus blood than in capillary blood during the first 2 weeks of life, while the number of erythrocytes in sinus blood is at first higher than and then equals that in capillary blood. A second possible factor is that with two exceptions the infants of this study were artificially fed. Other workers have studied chiefly infants fed human milk. It is well known that the iron of human milk is better retained by the infant than the same amount of iron in cow's milk. The blood values of the two infants of this study given human milk, were not conspicuously different from the values of the infants given cow's milk. The third and most obvious possible cause of the low hemoglobin values is the quantity of blood removed for study. As several factors were studied and the determinations were carried out in duplicate, 3 cc. samples of blood were taken. The total quantity of blood removed from an infant varied from 3 to 60 cc. After correction for the 20 to 25% of freed

iron which is excreted from the body, as will be discussed subsequently, the cumulative effect of several successive removals of these small amounts of blood is equivalent to the removal of sufficient iron to permit an average hemoglobin content at 20 weeks of age of from 0.3 to 1.0 gm. greater per 100 cc. of blood than the amount actually found. The removal of 3 cc. of blood weekly over a period of 6 to 20 weeks may thus be a real factor in depleting blood hemoglobin. Correcting for the average amounts of blood lost, the minimal hemoglobin values at 10 weeks would be 11.10 instead of 10.67 gm. per 100 cc. and the average value at 20 weeks, 12.27 instead of 11.80 gm. per 100 cc. These values approximate the averages found by others.

IRON BALANCE STUDIES

Table 2 shows the average daily intake, fecal excretion and loss of iron of seven infants between the ages of 11 and 57 days. The twenty-six periods of study varied from 1 to 11 days in length, averaging 4 days each. The iron intake varied from 0.64 to 1.01 mg. a day with a group average of 0.76 mg. daily. The average daily excretion for any single period varied from 0.92 mg. to 4.33 mg., and while each baby showed considerable variation in the amount of iron excreted, certain infants, notably D.A. and D.Sh., consistently excreted more iron than others. D.L. had been born prematurely, weighing 2458 gm. at birth. His average daily iron excretion was practically identical with the average excretion of the group.

In no period studied was the intake greater than the excretion. The smallest loss, 0.25 mg. was observed during the last period of study of R.P. at 1 month of age. The greatest daily loss was 3.60 mg. and the average for the entire group was 1.22 mg. daily.

The relative daily iron loss of an infant bore no apparent relation to the length of the period of blood destruction. The duration of this period varied markedly among the several infants, the extremes being 21 and 76 days, respectively.

TABLE 2

Daily iron balance of infants during period of hemoglobin destruction

NAME. AGE AT LOWEST BLOOD FE	AGE	AVERAGE WEIGHT	AVERAGE DAILY INTAKE	AVERAGE DAILY FECAL EXCRETION	AVERAGE DAILY LOSS
	days	*gm.*	*mg.*	*mg.*	*mg.*
R.P. 31 days	12–15	3495	0.71	1.63	0.92
	19–21	3700	0.62	1.62	1.00
	25–27	3930	0.69	1.35	0.66
	27–28	3960	0.71	3.35	2.64
	32–34	4297	0.69	0.94	0.25
Average			0.68	1.60	0.92
R.D. 36 days	11–14	3750	0.54	1.62	1.08
	17–19	3800	0.63	1.83	1.20
	23–25	3965	0.66	2.85	2.19
	29–30	4125	0.69	1.40	0.71
	37–39	4362	0.73	1.87	1.14
Average			0.64	1.94	1.30
H.E. 21 days	16–20	3975	0.70	1.14	0.44
	21–25	4155	0.81	1.97	1.16
	25–28	4317	0.62	1.32	0.70
	28–32	4425	0.63	2.82	2.19
Average			0.69	1.83	1.14
D.Sh. 40 days	20–28	3192	0.70	3.06	2.36
	28–30	3415	0.76	2.43	1.67
	30–31	3472	0.82	1.30	0.48
	31–38	3637	0.82	2.39	1.57
Average			0.76	2.63	1.87
D.A. 29 days	16–19	3167	0.62	4.22	3.60
	27–34	3642	1.07	2.19	1.12
	34–45	4027	1.07	2.36	1.29
Average			1.01	2.57	1.56
D.Sm. 76 days	25–32	4400	0.73	1.30	0.57
D.L. (prematurely born) 69 days	24–27	3005	0.71	3.08	2.37
	32–39	3380	0.80	1.52	0.72
	39–50	3735	0.82	1.68	0.86
	50–57	3992	0.83	2.51	1.68
Average			0.81	2.00	1.19
Group average			0.76	1.98	1.22

H.E., with the shortest period of hemoglobin destruction, 21 days, showed the relatively moderate daily loss of 1.14 mg. of iron. D.A. whose period of hemoglobin breakdown was completed at 29 days, lost 1.56 mg. of iron a day, while D.Sh., whose daily iron loss was the highest of the group, did not attain the minimal blood iron until the fortieth day.

Similarly, no consistent relationship was observed between the rate of hemoglobin destruction and the iron loss. The daily iron loss of D.Sh. was twice as great as that of R.P. yet the rate of decrease of hemoglobin was slower in D.Sh. than in R.P. Again R.P. showed a decreased loss of iron from the body after the blood iron reached its minimum, whereas the iron loss of infants D.A., H.E. and R.D. remained high.

One may conclude from the study of these infants, that a daily loss of approximately 1.25 mg. of iron from the body is to be expected in artificially-fed infants up to $1\frac{1}{2}$ to 2 months of age. Stearns and Stinger ('37), in this laboratory, found that infants over 2 months of age who were given cow's milk feedings, were approximately in iron equilibrium. An artificially-fed baby then during the first 2 months of life may be expected to lose daily approximately 1.25 mg. more of iron than he ingests but to approach equilibrium soon after that period. The total iron lost to the body would thus be from 50 to 75 mg.

The quantities of iron lost daily through the feces by this group of infants are greater than the amounts lost by the breast-fed infants studied by others. The greatest daily loss reported for an infant given human milk was 1.28 mg. (Langstein and Edelstein, '13), a value practically identical with the average daily loss of the babies of this study fed cow's milk. In six of the twenty-six periods studied here, the daily loss exceeded 2 mg. It thus appears that very young infants given cow's milk feedings do not conserve as much of the iron freed by hemoglobin breakdown as do infants fed human milk.

It is obvious that the amount of iron made available for storage by the breakdown of excess hemoglobin will depend upon several related factors, viz., the quantity of blood at

birth and its percentage of hemoglobin, the length of the period of hemoglobin destruction and the level to which the hemoglobin falls, and finally upon the ability of the infant to conserve the iron as it is freed. An infant whose hemoglobin is high at birth and whose period of iron loss is relatively short may be able to store from 250 to 300 mg. of iron from the excess hemoglobin. On the other hand, a premature infant, whose blood volume is small, if the loss of iron continues high for any considerable period, may conserve less than 50 mg. In general, it is probable that the average full-term infant will store from two to three times as much iron from the excess hemoglobin of blood as the maximum amount available in the liver at birth. No infant reported has been able to conserve all of the iron from the excess hemoglobin. If the average iron loss of the infant given cow's milk feedings is from 50 to 75 mg. during the first 2 months of life, it seems that some additional source of iron in the diet is desirable at an early age.

The amount of loss is perhaps more important than the amount stored. The theoretical iron content of the baby of 6 months is approximately equal to its iron content at birth.

SUMMARY

Alterations of cell volume, erythrocytes, hemoglobin and blood iron of infants under 2 months of age were studied in relation to the excretion of iron during this period.

Blood iron reached its minimal value between 4 and 6 weeks of age. The iron excretion of each infant studied during the period of decreasing blood iron was always greater than the intake. This loss of iron from the body continued for some time after the minimal blood iron was reached. The average daily loss was 1.25 mg., the estimated total loss for the period, from 50 to 75 mg. The one prematurely born infant studied showed as great a loss of iron from the body as did the full-term infants, who presumably had a much larger quantity of excess hemoglobin.

It is concluded that a dietary source of iron is desirable well before 6 months of age.

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A COMPARATIVE STUDY OF THE RESPIRATORY QUOTIENT FOLLOWING THE INGESTION OF GLUCOSE AND OF FRUCTOSE AS AFFECTED BY THE LACTIC ACID AND CARBON DIOXIDE CHANGES IN THE BLOOD

GEORGE BACHMANN AND JOHN HALDI

WITH THE TECHNICAL ASSISTANCE OF W. WYNN AND C. ENSOR

T. T. Fishburne Laboratory of Physiology, Emory University, Georgia

TWO FIGURES

While studies on the respiratory exchange have established with a fair degree of certainty the time, extent and duration of the rise of the respiratory quotient following the ingestion of glucose and of fructose, the significance of the quotient has been called into question by observations on lactic acid and carbon dioxide changes in the blood induced by the sugars. Katayama ('26) observed an increase in blood lactic acid after the ingestion of 1.75 gm. of glucose per kilogram body weight. Similar observations were made by Folch and Formiguera ('34) following the administration of 50 gm. of glucose dissolved in 250 cc. of water. Their data on normal subjects, however, showed an increase of only 0.4 mg., 2.5 mg. and 3.4 mg. %, ½ hour, 1 hour and 2 hours, respectively, after the ingestion of the sugar. Mendel, Engel and Goldscheider ('25), on the other hand, found that the blood lactic acid remained constant after giving by mouth 50 or 70 gm. of glucose; similarly, Oppenheimer ('28) using Mendel and Goldscheider's method for lactic acid analysis, found no increase in the lactic acid of the blood of normal subjects or of patients with diseases of the liver after administration of 50 gm. glucose or of fructose

by mouth. Campbell and Maltby ('28) noted that the ingestion of 100 gm. of fructose was followed by an increase in the lactic acid with a concomitant decrease in the carbon dioxide capacity of the blood, whereas the ingestion of the same amount of glucose had no effect. The oral administration of 50 gm. of glucose to a dog (Taistra, '21) had no effect on the carbon dioxide combining power of the blood. No change in the lactic acid of the blood could be found by Lanyi (cited by Carpenter and Lee, '33) after the administration of glucose to dogs or to man, while the ingestion of fructose was followed by an increase in blood lactic acid, lasting 3 hours. Rose, Giragossintz and Kirstein ('30) injected 25 gm. of glucose or fructose in 125 cc. of water into the small intestine of a dog under amytal anesthesia. The lactic acid in the portal vein rose between 50 and 100% above the resting values in all fructose experiments but showed little or no change with the injection of glucose. The continuous intravenous injection of glucose and of fructose into dogs was found by Wierzuchowski and Laniewski ('31) to lead to an increase in blood lactic acid which was much greater with fructose than with glucose. A rise in the blood lactic acid of rabbits was observed by Koike ('34) after intraperitoneal injections of glucose or fructose.

These experiments would suggest that the respiratory quotient following the ingestion of glucose and of fructose may be affected by the displacement of carbon dioxide from blood bicarbonate as a result of the increase in blood lactic acid. Because of the contradictory observations on lactic acid changes in the blood induced by these sugars, Carpenter and Lee ('33) made simultaneous observations on the respiratory quotient and the carbon dioxide percentage of the alveolar air following the ingestion of glucose and of fructose, to determine whether or not there is a loss of non-metabolic carbon dioxide from the blood. They observed that with a trained subject there was no change in the alveolar carbon dioxide accompanying the rise in the respiratory quotient after the ingestion of either of the two sugars. On the premise,

frequently suggested in the literature, that the constancy of the alveolar air is an indication of true metabolic respiratory quotients, they concluded that no organic acid was formed and that the rise in the respiratory quotient must consequently be regarded as the result of the metabolism of the sugar. As it is questionable whether the constancy of the alveolar carbon dioxide can be regarded as an index of true metabolic quotients, it was deemed advisable to reinvestigate the problem by the more direct method of determining the lactic acid and carbon dioxide content of the blood, and to correlate these findings with the respiratory quotient.

METHOD

The gaseous exchange was determined by the open circuit method of Carpenter and Fox ('31) with some modifications.[1] The principle of the apparatus used in this method as stated by Carpenter and Fox

is that of the open circuit type in which the expired and inspired air are separated by valves; the expired air passes into a spirometer, from which it is continuously removed by a blower, then passes by a sampling device, and is finally measured by a meter.

The apparatus admits of the measurement of expired air over an extended period, which may be divided into shorter periods of any practical duration. It is adapted to the study of the respiratory exchange both during rest and muscular work, and has a distinct advantage in that the movements of the spirometer bell, which are used to record the character of the breathing on a kymograph paper, take place without the necessity of an increased force of expiration on the part of the subject. The technic of manipulation is easily acquired.

A diagram of the system is presented in figure 1. In our apparatus, provision has been made for the attachment of two sampling bags instead of one, as in the set-up described by Carpenter and Fox. With this arrangement it is possible to

[1] We wish to acknowledge our indebtedness to Doctor Carpenter for his kindly advice at the beginning of the work concerning the construction and use of his respiration apparatus.

cut off one of the sampling bags and to connect the other simultaneously with the main stream of gas flowing through the system.

The original plan was to use basket-ball bladders for collecting the gas samples as suggested by Carpenter and Fox. With this size bag it is necessary to diminish the rate of flow so as to prevent undue distension of the bag and a consequent back pressure which would interfere with aliquot sampling. For this purpose a metal disc[2] with a small opening (G in fig. 1), as recommended by Simonson ('29), was inserted between the main stream of expired air and the sampling bag.
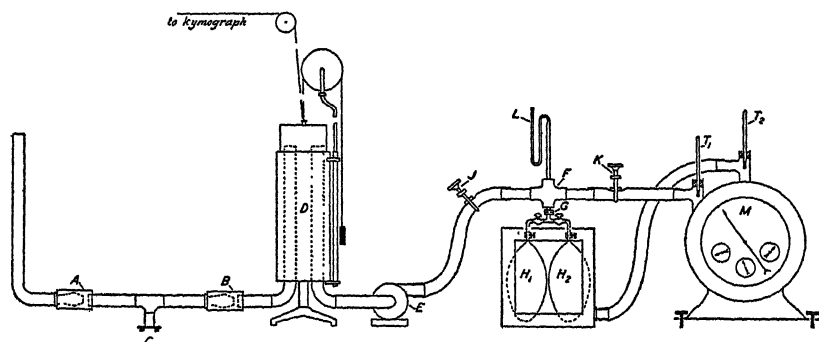
Fig. 1 Schema of the respiration apparatus (after Carpenter and Fox). A, inspiratory valve; B, expiratory valve; C, mouthpiece; D, spirometer; E, rotary blower; F, cross through which expired air passes to the manometer L and into the union G which holds a perforated disc; H_1 and H_2, sampling bags; M, meter; T_1 and T_2, thermometers; J and K, screw clamps.

Pressure in the main stream was adjusted by two screw clamps (J and K) as recommended by Carpenter and Fox, thereby regulating the amount of gas collected in the sampling bag.

In checking the apparatus it was found that the pencentage composition of a gaseous mixture is altered when it passes through an aperture less than 1 mm. in diameter. This source of error was discovered by the following procedure: A Tissot spirometer was filled with a mixture of carbon dioxide and oxygen and its exact composition determined by analysis.

[2] Discs with various sized apertures ranging from 0.1 to 0.7 mm. in diameter can be obtained from the Askania-Werke A. G. of Berlin, through their American representative, The American Askania Corporation, Houston, Texas.

The spirometer was then connected to the respiration apparatus and its contents drawn through the system by the blower E (fig. 1). Analysis of the gas mixture that came out of the meter showed that its composition had not been altered in passing through the system; yet the oxygen percentage of the mixture collected in the sampling bag was increased approximately 0.13% and the carbon dioxide percentage decreased approximately 0.20% in passing through an aperture 0.4 mm. in diameter.

The different percentage composition of the gas mixture in the main stream and in the sampling bag after passing through a small aperture, may perhaps be accounted for by the different rates of effusion of oxygen and carbon dioxide. That the different gases have a different rate of effusion is shown by Graham's law which states that, when two different gases are forced through a small aperture, the times t_1 and t_2 taken for each gas to pass through under a given pressure difference are inversely proportional to the velocities of the gas molecules, according to the formula,

$$\frac{t_1}{t_2} = \frac{u_1}{u_2} = \sqrt{\frac{M_1}{M_2}}$$

where u_1 and u_2 stand for the average velocities, and M_1 and M_2, the molecular weights of the two gases.

It is important to note that with alcohol checks it is possible to obtain from gas analyses respiratory quotients close to the theoretical and yet have an appreciable error in the calculated total oxygen consumption and carbon dioxide evolution. In our early work on the method in which we were using a disc with two pin holes which we had made ourselves, alcohol checks yielded respiratory quotients of 0.656, 0.672 and 0.669 which were regarded as fairly satisfactory; the error in the volume of oxygen consumed and carbon dioxide produced, calculated from our analyses of the gas sample was, however, beyond the limit of experimental error. Further experimentation showed that there was an increase of 0.17% in the oxygen and a decrease of 0.16% in the carbon dioxide of the gaseous mixture after it had passed into the sampling bag through the small apertures. If this error had occurred

in the first experiment of table 1 in which are given the results of alcohol checks obtained with a 1.04 mm. aperture, it would have produced only a small change in the respiratory quotient, but a large difference in the oxygen consumption and carbon dioxide production. In this experiment the calculated oxygen consumption was 4.942 liters and carbon dioxide evolution 3.248 liters, giving a respiratory quotient of 0.657. The percentage oxygen and carbon dioxide in the sampling bag (not given in the table) were, respectively, 16.550 and 3.132. If we had had the error referred to above, the oxygen percentage would have been 16.720 and the carbon dioxide percentage 2.972. The customary calculations would then have yielded an oxygen consumption and carbon dioxide production, respectively, of 4.760 and 3.081 liters or a respiratory

TABLE 1

Alcohol checks with respiration apparatus, using large sampling bags and a disc with an aperture 1.04 mm. in diameter

TIME	C_2H_5OH BURNED	AIR PASSED THROUGH SYSTEM	CHANGES IN COMPOSITION OF AIR		FOUND		EXPECTED		ERROR		R.Q.
			CO_2 increase	O_2 decrease	Total CO_2 produced	Total O_2 consumed	Total CO_2 from combustion of C_2H_5OH	Total O_2 consumed in combustion of C_2H_5OH	Total CO_2	Total O_2	
min.	gm.	liters	%	%	liters	liters	liters	liters	%	%	
15	3.371	104.385	3.112	4.734	3.248	4.942	3.280	4.920	—0.97	+0.45	0.657
15	3.306	87.281	3.646	5.518	3.182	4.816	3.216	4.825	—1.06	—0.19	0.661
15	3.322	89.442	3.580	5.406	3.202	4.835	3.232	4.848	—0.93	—0.27	0.663

quotient of 0.647 as compared with the respiratory quotient of 0.658 that was actually obtained. The error in the total volume of oxygen consumed, however, would have been 3.7% and in the total volume of carbon dioxide evolved, 5.1%. It was therefore necessary for accurate results to use an aperture larger than 1 mm. in diameter. With an aperture of this size a basket-ball bladder would become filled and distended within a few minutes, thereby necessitating collection of gas samples over correspondingly short intervals. In many metabolism experiments it suffices to collect gas samples over intervals of 15 minutes. After considerable experimentation in which various methods were tried for collecting gas samples, we adopted as the most satisfactory procedure the use of rubber bags large enough to contain approximately 10 liters of gas without exerting a back pressure. A bag of this size

is satisfactory for experiments such as we have been conducting in which the maximum ventilation was from 25 to 30 liters per minute. Using this size bag and a disc with an aperture 1.04 mm. in diameter, we have found in a series of tests with a minimum flow of gas of 6 liters and a maximum of 28 liters per minute, that the bag fills at a uniform rate throughout a 15-minute period. A uniform rate of filling is necessary to make certain that the gas in the sampling bag is an aliquot sample of the gas mixture passing through the system. The bags we used were 46 cm. wide and 55 cm. long. They are prevented from kinking when suspended in the box by two paper clips attached by a wire from the under surface of the top of the box. This arrangement also serves as a safeguard against undue traction by the sampling bag on the rubber tube to which it is cemented.

The rubber sampling bags which we are now using were constructed according to our specifications by a local rubber company.[3] They can, however, be made by any laboratory worker. Two sheets of ordinary cold patching are held together and cut to the desired size and shape. Cold patching 0.046 inch (1.18 mm.) in thickness was used in the bags made for us, but we have found that thinner rubber (0.031 inch, or 0.787 mm.) is satisfactory. When the cold patching has been cut, the partially cured surface of each piece is covered with a thin coat of talc, with the exception of an outer rim about ¾ inch wide. The outer rim having been cleaned with benzol, is covered with a thin layer of rubber cement. A short piece of gum tubing is placed in the proper position to serve as an inlet for the gas and the two sheets of rubber glued together. A small collar is made from the cold patching and a hole cut in it, the exact size of the rubber tubing. The collar is slipped over the tube and fastened by cement to the rim of the bag. A copper tube of 5 mm. internal diameter is fastened into the rubber tubing. A 'gasoline line' nipple and coupling at the free end of the copper tubing serve to make an air-tight connection between the sampling bag and respiration apparatus.

The results obtained with alcohol checks using a disc with a 1.04 mm. aperture and large rubber bags for collecting the samples are given in table 1. The accuracy of the method has been further tested by simulating experimental conditions in which alterations occur in the oxygen and carbon dioxide

[3] The Holfast Rubber Company, Atlanta, Ga.

percentage of the expired air passing through the system. A Tissot spirometer was connected by a T-tube with the respiration apparatus. A rubber tube led off from the arm of the T-tube to a calibrated 8 liter aspirator bottle. The gas in this bottle could be displaced and forced into the T-tube by introducing acidulated water from another aspirator bottle, the flow of water being controlled by a stopcock interposed in the tubing connecting the two bottles. The Tissot spirometer and aspirator bottle were filled with different gas mixtures, the exact composition of which was determined by analysis. The flow of gas from either of the containers could be interrupted at will by clamping the rubber tubing near the spirometer or aspirator bottle. A flutter valve placed in the path of flow between the Tissot spirometer and T-tube prevented the gas from the aspirator bottle flowing backward into the spirometer. Gas was first delivered from the Tissot spirometer, then from the aspirator bottle and again from the spirometer, thereby altering in the course of the experiment the percentage composition of the gas flowing through the system. During the first and last few minutes of the experiment, gas was delivered from the spirometer so that the percentage composition of the gas in the system was the same at the end as at the beginning of the experiment. As the total volume and percentage composition of the gas mixture were known, it was an easy matter to determine the exact amount of oxygen and carbon dioxide that had passed through the system. The total oxygen and carbon dioxide were then calculated from the oxygen and carbon dioxide percentage of the gas mixture in the sampling bag in the same manner as in a metabolism experiment. The volume of the two gases thus calculated was almost identical with the known volume that had passed through the system. In most instances the difference was less than $\frac{1}{2}\%$. The results of this experiment are given in table 2.

The sample of gas for analysis is drawn from the large rubber bag into a well oiled 50 cc. Vim syringe. Connection between the sampling bag and syringe is made by slipping a

TABLE 2

Checks on respiration apparatus using large sampling bag and disc with 1.04 mm. aperture. Gas mixtures of different percentage composition were delivered alternately from two containers

KNOWN GAS MIXTURES PASSED THROUGH THE APPARATUS — FOUND

No.	103.55 liters delivered from large spirometer				7.00 liters delivered from small container				Total volume delivered from the two containers		Sample in bag		Total volume of gas as calculated from sample		Error in total volume	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
	%	%	liters	liters	%	%	liters	liters	liters	liters	%	%	liters	liters	%	%
1	19.364	2.405	20.051	2.490	17.158	5.393	1.201	0.378	21.253	2.867	19.169	2.585	21.191	2.858	−0.288	−0.356
2	19.405	2.435	20.094	2.521	15.666	10.093	1.097	0.707	21.191	3.228	19.150	2.903	21.170	3.209	−0.100	−0.580
3	19.007	2.972	19.682	3.078	16.229	8.307	1.136	0.582	20.818	3.659	18.856	3.260	20.845	3.604	+0.130	−1.500

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rubber tube fastened to the syringe, over the copper tube leading into the bag. The gas is 'pumped' back and forth into the bag several times so as to mix thoroughly the small amount of air in the dead space of the rubber tube with the gas in the bag. The syringe is then filled and the rubber tube closed tight by means of a screw clamp. Two or 3 cc. of mercury in the bottom of the syringe serve as an extra precaution against escape of the gas, while the syringes are standing in a rack until the gases are analyzed. The volume of the gas in the bag is measured by displacement of water from an 8 liter calibrated aspirator bottle. This volume is added to the amount of gas that has passed through the meter.

EXPERIMENTAL PROCEDURE

The subjects of these experiments, three male adults, weighing 80, 70 and 55 kilo., respectively, came to the laboratory in the fasting state, between 7.15 and 7.45 in the morning, the last meal having been taken not later than 7 o'clock the evening before. Upon arriving at the laboratory the subject reclined on a couch for 30 minutes to recover from the effects of previous exertion, which he had endeavored to keep at a minimum. After this preliminary rest period, the mouth-piece commonly employed in metabolism experiments was inserted, and the subject then breathed into the respiratory apparatus for 45 minutes, during which time the 'post-absorptive' gaseous exchange was determined, gas samples being collected over 15-minute period intervals. At the conclusion of the post-absorptive period a sample of venous blood was drawn from the arm without stasis. A portion of the blood was then delivered from the syringe into chilled test tubes under oil for determinations of its carbon dioxide content. Coagulation was prevented by the addition of 1 mg. potassium oxalate per cubic centimeter of blood. The remainder of the blood was used for lactic acid analysis. Glycolysis was inhibited in this portion of the blood by the addition of 10 mg. of sodium fluoride per cubic centimeter of blood. The samples for carbon dioxide determinations were surrounded by ice, and the analyses

usually completed within 2 to 3 hours after withdrawal of the blood. Preliminary tests had shown that there was no loss of carbon dioxide during this time when the blood was kept under these conditions.

The gas analyses were made by two analysts using the Haldane-Henderson method. Checks within 0.02% were required. The lactic acid of the blood was determined by the Friedemann, Cotonio and Shaffer ('27) method, and its carbon dioxide content by the Van Slyke procedure (Peters and Van Slyke, '32).

After the withdrawal of the post-absorptive blood sample, 500 cc. water, or 50 gm. glucose, 50 gm. fructose or 25 gm. each of glucose and fructose in 500 cc. of water at 37°C. were ingested, the experiments with water serving as controls. The subject then resumed the recumbent position and the determination of the gaseous exchange proceeded in the same manner as during the post-absorptive period. Gas samples were collected over 15-minute periods, except in a few experiments to be referred to later, in which the intervals were shorter. Blood samples were drawn at various times. An attempt was made to obtain the sample at the end of that 15-minute period which we had found by previous experience usually showed the highest respiratory quotient. In this we were only partially successful as was to be expected, since the highest respiratory quotient was not always obtained in the same 15-minute period; in the glucose experiments, the maximum quotient was observed sometimes in the third, sometimes in the fourth 15-minute period, whereas in the fructose experiments the highest quotient was obtained sometimes in the second and sometimes in the third period.

RESULTS

As seen in table 3, the control experiments with water carried out in the same way as the sugar experiments, showed no increase in the lactic acid or decrease in the carbon dioxide content of the blood after the ingestion of 500 cc. water. In one experiment in which the lactic acid remained constant,

TABLE 3

Lactic acid and carbon dioxide content of the blood, before and after ingestion of water (control experiments), glucose and fructose

EXPERIMENT NO.	SUBSTANCE INGESTED	NON PROTEIN R.Q.[5]		BLOOD LACTIC ACID		CO₂ CONTENT OF BLOOD		15 MINUTES POST-INGESTION PERIOD[1] IN WHICH SECOND BLOOD SAMPLE WAS DRAWN
		Post-absorptive	Period[1] before drawing second blood sample	End of post-absorptive periods[1]	Second sample	End of post-absorptive periods[1]	Second sample	
				mg. %		volume %		
1	500 cc. water	0.83	0.82	....	....	57.6	58.0	End of 3rd
2	500 cc. water	0.85	0.83	11.2	10.4	60.0	62.0	End of 3rd
3	500 cc. water	0.84	0.84	10.8	10.7	61.6	61.0	End of 3rd
4	500 cc. water	0.81	0.79	8.7	7.9	57.8	57.5	End of 3rd
5	50 gm. glucose	0.78	0.78	10.3	7.4	54.6	54.1	End of 3rd
6	50 gm. glucose	0.78	0.83	14.6	15.3	60.2	56.7	End of 3rd
7	50 gm. glucose	0.76	0.85	9.4	9.4	....	....	End of 3rd
8	50 gm. glucose	0.75	0.85	14.6	14.1	59.8	59.8	End of 3rd
9	50 gm. glucose	0.78	0.86	8.9	9.8	54.9	55.3	End of 3rd
10	50 gm. glucose	0.80	0.90	6.3	11.0	58.9	54.9	End of 4th
11	50 gm. fructose	0.80	1.05	9.3	14.5	60.1	54.1	End of 2nd
12	50 gm. fructose	0.78	0.96	13.8	25.8	60.6	53.6	End of 2nd
13	50 gm. fructose	0.79	0.95	7.3	16.5	56.1	52.0	End of 3rd
14	50 gm. fructose	0.78	0.95	13.8	21.2	56.0	54.0	End of 3rd
15	50 gm. fructose	0.82	0.98	9.3	14.7	....	....	End of 3rd
16[2]	50 gm. fructose	0.77	0.86	9.1	16.2	58.6	54.5	End of 1st
17[3]	50 gm. fructose	0.78	0.96	10.8	15.2	61.9	58.6	End of 1st
18	25 gm. glucose 25 gm. fructose	0.78	0.88	....	....	60.1	58.3	End of 4th
19	25 gm. glucose 25 gm. fructose	0.77	0.96	8.6	15.5	56.7	56.4	End of 2nd
20	25 gm. glucose 25 gm. fructose	0.76	1.03	20.8	33.0	59.2	54.4	End of 2nd
21	25 gm. glucose 25 gm. fructose	0.76	0.93	7.5	15.9	56.5	55.9	End of 3rd
22	25 gm. glucose 25 gm. fructose	0.75	0.87	17.8	26.9	59.6	56.1	End of 3rd
23	25 gm. glucose 25 gm. fructose	0.78	1.02[4]	9.1	19.9	59.8	55.1	Middle of 3rd

[1] Periods were of 15 minutes duration.

[2] A third blood sample drawn at the middle of the fourth 15-minute period contained 24.4 mg. % lactic acid and 52.0 volumes % carbon dioxide. The non-protein respiration quotient for the 8-minute period immediately before the withdrawal of the blood was 1.04.

[3] A third blood sample, drawn at the middle of the third 15-minute period, contained 20.6 mg. % lactic acid and 54.0 volumes % carbon dioxide. The non-protein respiratory quotient for the 7-minute period, immediately before the withdrawal of the blood, was 1.08.

[4] Ten-minute period.

[5] The urine was analyzed for nitrogen in each experiment for the calculation of the non-protein respiratory quotient.

there was an increase in the carbon dioxide content of the second blood sample. We are at a loss to account for this irregularity as perfect checks were obtained on the carbon dioxide analyses. It is possible that in spite of the precautions observed there was a slight stasis during the withdrawal of the second sample.

In the glucose experiments, the blood lactic acid remained constant in five out of six experiments; one showed an increase of 4.7 mg. % with a concomitant decrease of 5 volumes % in the carbon dioxide content. The blood sample in this experiment was drawn 15 minutes later than in the others. In the remainder of the experiments, the carbon dioxide content of the blood remained constant except in one instance (experiment no. 6) when it fell 3.5 volumes % although there was no change in the blood lactic acid. Irregularities of this nature are found also in the results reported by Campbell and Maltby ('28).

In the fructose experiments, there was in each instance an increase in the lactic acid of the blood drawn 30 to 45 minutes after the ingestion of the sugar, and a uniform decrease in the carbon dioxide content of the blood. The decrease in the carbon dioxide content was greater than one might expect from a complete reaction of lactic acid with bicarbonate since 2.5 cc. of carbon dioxide would have been displaced by each 10 mg. of lactic acid entering into the reaction. This suggests that there may have been some other fixed acid or acids formed as intermediate products in the metabolism of fructose.

After the ingestion of the mixture of the sugars there was an increase in the blood lactic acid in all the experiments and a decrease in the carbon dioxide content of the blood in all but two experiments. Since there was no increase in the lactic acid in the glucose experiments, the increase induced by the mixture of the sugars must be attributed to the action of fructose. It is interesting to note that the average lactic acid and carbon dioxide changes of the blood were approximately the same after the ingestion of 50 and 25 gm. of fructose.

It should be noted that the lactic acid increase occurred within a short time after the ingestion of fructose. In experiments 16 and 17 the lactic acid had risen 7.1 and 4.4 mg., respectively, 15 minutes after ingestion of the sugar while the carbon dioxide had fallen 4.1 and 3.3 volumes % (fig. 2). During this time, the respiratory quotient had risen to 0.85 and
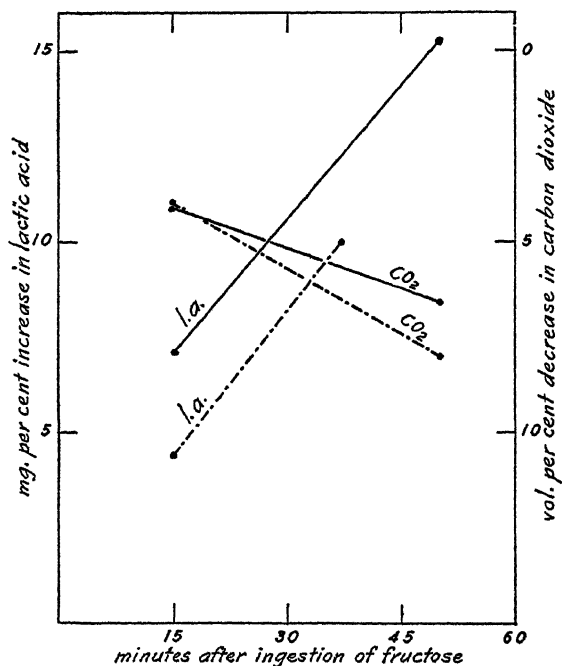
Fig. 2   Changes in the lactic acid and carbon dioxide of the blood in two subjects after the ingestion of fructose.

0.92, respectively, which was an increase of 0.11 and 0.13 over the post-absorptive level. In the same period after the ingestion of glucose the average rise of the respiratory quotient was only 0.01 or 0.02.

From these results, it is obvious that the character of the metabolism of fructose is masked by the blowing off of non-metabolic carbon dioxide. In order then to interpret the

findings of the respiratory exchange correctly and to draw a comparison between the respiratory quotients obtained after the ingestion of glucose and of fructose, it becomes necessary to correct the respiratory quotients of the fructose experiments by making proper allowance for the volume of non-metabolic carbon dioxide blown off.

The correction of the respiratory quotients obtained for the time elapsing between the ingestion of the sugar and the drawing of the blood sample has been made in two ways: 1) By deducting from the total carbon dioxide eliminated the amount that would be liberated by the lactic acid that was formed after the ingestion of fructose, 2) by deducting an amount equivalent to the loss of carbon dioxide from the blood as calculated from the reduction in its carbon dioxide content. The results of these calculations in the form of 'corrected' respiratory quotients are given in table 4. The total volume of blood has been estimated as 7% of the body weight of the subject and 1 mg. of lactic acid taken as the equivalent of 0.25 cc. of carbon dioxide. The calculations were made on the assumption that there was a complete reaction between the lactic acid and bicarbonate of the blood. This perhaps is too generous an allowance, for Mellanby and Thomas ('20-'21) found that the addition of lactic acid to blood in vitro diminishes its carbon dioxide capacity by less than 50% of the quantity demanded by complete reaction with bicarbonate. Similar results were also obtained by Evans ('22). In the experiments of Gesell, Krueger, Gorham and Bernthal ('30) it appears that approximately 40% only of the lactic acid formed during the administration of low oxygen, combined with the bicarbonate of the blood. By making the maximum allowance for displacement of carbon dioxide by the increased lactic acid, the question is therefore put to the most severe test. If our calculations had been made on the assumption that only 40 to 50% of the lactic acid reacted with bicarbonate, there would have resulted a considerably smaller difference between the corrected and actual respiratory quotients.

The respiratory quotients in the last column of table 4 represent the average quotients obtained from a number of experiments on the different subjects after the administration

TABLE 4

Respiratory quotients after fructose ingestion, corrected for blowing off of non-metabolic carbon dioxide, and compared with respiratory quotients obtained with glucose over corresponding periods of time

EXPERIMENT NO.		TIME BETWEEN INGESTION OF SUGAR AND DRAWING OF BLOOD SAMPLE	INCREASE IN BLOOD LACTIC ACID	DECREASE IN CO₂ CONTENT OF THE BLOOD	NON-PROTEIN RESPIRATORY QUOTIENT			
					Obtained for time between ingestion of sugar and drawing of blood sample	Corrected for CO₂ equivalent of lactic acid increase	Corrected for CO₂ equivalent of decrease in blood CO₂	Of corresponding period in glucose experiments
		minutes	*mg. %*	*vol. %*				
16	(Increase over basal)	15	5.0	4.2	0.864	0.851 (0.086)[1]	0.826 (0.061)	0.777 (0.011)
11	(Increase over basal)	30	5.2	6.0	0.932	0.920 (0.122)	0.875 (0.077)	0.822 (0.033)
15	(Increase over basal)	30	12.0	7.0	0.948	0.938 (0.166)	0.914 (0.142)	0.760 (0.000)
17	(Increase over basal)	30	4.5	3.3	0.931	0.908 (0.128)	0.880 (0.100)	0.760 (0.000)
12	(Increase over basal)	45	9.2	4.1	0.853	0.843 (0.114)	0.836 (0.107)	0.808 (0.052)
13	(Increase over basal)	45	7.4	2.0	0.907	0.899 (0.121)	0.898 (0.120)	0.808 (0.052)

[1] The post-absorptive quotients (not included in the table) can be obtained by subtracting the increase over the basal enclosed in parentheses from the figure immediately above.

of glucose, except that of experiment 17 which is the respiratory quotient of only one experiment on this subject with glucose. The respiratory quotients of the glucose and those of the fructose experiments with which they are compared, were obtained from the respiratory exchange of the same

subject, over the same length of time and under similar experimental conditions.

It will be observed in the table that after the maximum allowance has been made for the blowing off of non-metabolic carbon dioxide in the fructose experiments, the respiratory quotients for 15-, 30- and 45-minute periods after ingestion of the sugar, are still higher than those obtained in the glucose experiments over the same length of time. The increase in the respiratory quotient over that of the post-absorptive periods is given in the table in parentheses. The post-absorptive quotients, which are not included in the tables, can be obtained by subtracting the increase enclosed in parentheses from the figure immediately above. From these observations we may conclude that the difference between the respiratory quotients observed after the ingestion of glucose and of fructose cannot be entirely accounted for by the formation of acids and elimination of the non-metabolic carbon dioxide that follow the ingestion of fructose. The difference must lie either in the combustion of relatively more carbohydrate during the first 45 minutes after the ingestion of fructose or in the transformation of this sugar into fat.

Respiratory quotients above unity which have been observed by a number of workers after the ingestion of fructose, have been offered as evidence of the conversion of this sugar into fat. Conceivably, these quotients might be reduced to unity or below when a correction is made for the blowing off of non-metabolic carbon dioxide. The ideal procedure would be to draw the blood sample at the time when the respiratory quotient is above unity. Such was our original plan at the outset of these experiments, as it was believed that the high respiratory quotients we had observed in earlier experiments could be easily duplicated. Unfortunately, these high quotients did not recur except in a few experiments (nos. 11, 16, 17, 20 and 23 of table 1). In two of these experiments (16 and 17), the high quotients were obtained by collecting the gas sample over 5- and 7-minute periods, respectively, instead of the usual 15-minute period.

The experiments with respiratory quotients above unity are given in table 5. This table gives also the quotients derived by correcting the original quotients for the carbon dioxide equivalent of blood lactic acid, and for the equivalent of the decrease in carbon dioxide content of the blood. In experiments 16 and 17 three blood samples were taken. In both experiments, the first sample was drawn at the end of the

TABLE 5

Respiratory quotients above unity corrected for non-metabolic carbon dioxide in the expired air

EXPERI-MENT NO.	SUGAR INGESTED	TIME (AFTER INGESTION) OVER WHICH R.Q. WAS DETERMINED	NON-PROTEIN RESPIRATORY QUOTIENT		
			Obtained from gaseous exchange	Corrected for CO_2 equivalent of increase in blood lactic acid	Corrected for CO_2 equivalent of decrease in blood CO_2
		minutes			
16	50 gm. fructose	37 to 45	1.040	1.031	1.023
17	50 gm. fructose	30 to 37	1.075	1.059	1.020
11	50 gm. fructose	30 to 45	1.050	1.011	0.997
20	25 gm. glucose 25 gm. fructose	25 to 30	1.094	1.070	1.054
23	25 gm. glucose 25 gm. fructose	25 to 35	1.019	1.005	0.986
61	50 gm. fructose	15 to 30	1.052	1.042	1.005
61	50 gm. fructose	30 to 45	1.088	1.073	1.019
62	50 gm. fructose	30 to 45	1.067	1.055	1.010
63	25 gm. glucose 25 gm. fructose	15 to 30	1.073	1.062	1.021
64	25 gm. glucose 25 gm. fructose	45 to 60	1.068	1.054	1.011
65	25 gm. glucose 25 gm. fructose	45 to 60	1.056	1.045	1.005

post-absorptive periods and the second sample 15 minutes after the ingestion of the fructose. In experiment 16 the third sample was taken 30 minutes, and in experiment 17, 22 minutes after the first sample. As seen in figure 2, these experiments indicate that the formation of lactic acid and the decrease in the carbon dioxide of the blood began shortly after the ingestion of the sugar and continued at a fairly uniform rate.

In experiments 11, 20 and 23, only two blood samples were taken, one at the close of the post-ingestion periods and the other 30 to 45 minutes after ingestion of the sugar. On the basis of the observations in experiments 16 and 17, all calculations of the increase in lactic acid, or decrease in carbon dioxide content of the blood for the time over which the respiratory quotient was determined were made on the assumption of a uniform rate of change.

It will be observed that in every instance the corrected respiratory quotients still remain above unity. The correction for lactic acid formation lowers the quotient to a slighter extent than that for the carbon dioxide decrease of the blood. This is in accordance with the observation mentioned previously that the decrease in the carbon dioxide of the blood was greater than could be accounted for by the rise in lactic acid.

In experiments carried out before the present investigation was undertaken, respiratory quotients above unity had been obtained several times after the ingestion of fructose and a mixture of glucose and fructose. Since, however, the same subject served in the present series, the early experiments (nos. 61 to 65) showing respiratory quotients above unity, have been incorporated in table 5. The correction of the quotients was made on the assumption that the quantitative changes in the lactic acid and carbon dioxide content of the blood were approximately the same, and occurred at the same rate as subsequently observed in other experiments on this subject after the ingestion of fructose. The quotients thus corrected as in the case of the experiments of the present series, are still above unity.

CONCLUSIONS

The respiratory quotients obtained within 45 minutes after the ingestion of 50 gm. fructose are not true metabolic quotients, for, as shown in these experiments there is a gradual rise in blood lactic acid and a decrease in the carbon dioxide content of the blood, beginning within a short time after the ingestion of the sugar. When, however, correction was made

for the non-metabolic carbon dioxide in the expired air, the respiratory quotients obtained after the ingestion of fructose in a number of experiments were still above unity. It must therefore be concluded that a portion of the ingested fructose was converted into fat.

As regards glucose, it would appear from our experiments that the respiratory quotients obtained over at least the first 30 minutes after the ingestion of the sugar, are true metabolic quotients.

SUMMARY

A study has been made of the respiratory quotient correlated with blood lactic acid and the carbon dioxide content of the blood, following the ingestion of 50 gm. glucose, 50 gm. fructose and a mixture of 25 gm. glucose and 25 gm. fructose.

The respiratory exchange was determined by the open circuit method of Carpenter and Fox. It was found that an error was introduced by using discs with a small aperture (less than 1 mm. in diameter), as recommended by Simonson, for collecting aliquot samples of the expired air. The percentage composition of the gas was altered in passing through the small aperture; the gaseous mixture in the sampling bag had a higher oxygen and lower carbon dioxide percentage than that of the main gaseous stream passing through the system. When the gases passed through an aperture 1.04 mm. in diameter, the percentage composition of the mixture in the sampling bag was identical with that of the main stream.

Large rubber bags, that contain approximately 10 liters of gas without exerting a back pressure, proved satisfactory for collecting gas samples through an aperture 1.04 mm. in diameter. A detailed account is given of the construction of the rubber bags used in these experiments.

In five experiments in which 50 gm. glucose was ingested there was generally no increase in the lactic acid nor decrease in the carbon dioxide content of the blood. It was concluded that the respiratory quotients obtained within 30 minutes after the ingestion of glucose were true metabolic quotients.

The ingestion of the same quantity of fructose led in each instance, within 15 minutes and later, to an increase in blood lactic acid and an attendant decrease in the carbon dioxide content of the blood. The same events followed the ingestion of a mixture of 25 gm. glucose and 25 gm. fructose. The average lactic acid and carbon dioxide changes observed with this mixture, were approximately of the same magnitude as those observed with 50 gm. fructose. From these observations, it is evident that the respiratory quotient obtained after the ingestion of fructose is not a true metabolic quotient.

When allowance was made for the blowing off of non-metabolic carbon dioxide, the respiratory quotients obtained over a period of 30 to 45 minutes after the ingestion of fructose, were considerably higher than the quotients obtained over the corresponding time after the ingestion of glucose. The difference indicates either a combustion of relatively more carbohydrate after the ingestion of fructose, or a transformation of this sugar into fat.

When the respiratory quotients obtained with fructose were above unity and these high quotients were corrected for the non-metabolic carbon dioxide, as calculated from the increase in the lactic acid and from the decrease in the carbon dioxide content of the blood, they still remained above unity. It is therefore concluded that a portion of the ingested fructose was converted into fat.

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EFFECT OF DIET ON THE CONSTANCY OF THE URINARY NITROGENOUS CONSTITUENTS EXCRETED DAILY BY PRE-SCHOOL CHILDREN [1]

JEAN E. HAWKS, MERLE M. BRAY AND MARIE DYE

*Section of Home Economics, Michigan Agricultural Experiment Station,
East Lansing, Michigan*

ONE FIGURE

Although investigators have determined the distribution of nitrogen in the urine, few studies have been made on normal pre-school children who have received constant diets for long periods of time. The two metabolic studies conducted in this laboratory on six normal pre-school children (Hawks, Bray and Dye, '37) included daily urinary analysis for total nitrogen, urea, uric acid, ammonia, creatine, creatinine and amino acid nitrogen. There were two children in the first study and four in the second. This paper presents the data obtained, following a 10- or 12-day preliminary period, during 21 consecutive days when the children received diets containing 3 gm. of protein per kilogram of body weight, and also the data obtained during the following 15 or 24 days when they received diets containing 4 gm. of protein per kilogram. Thus, the data show, first, the daily variations in the urinary nitrogenous substances when the children received a constant diet; second, the variations which occurred when the protein content of the diet was increased; third, the length of time necessary for the children to adjust to the change in diet; and fourth, the individual differences among the children.

The complete experimental procedure was described in detail in an earlier communication (Hawks, Bray and Dye, '37). In order to prevent changes in the acidity and in the distribution of the urinary nitrogenous substances, the urine samples were all kept on ice until the 24-hour sample was complete. They were then taken to the laboratory, adjusted to room temperature, made to a definite volume and analyzed immediately for acidity, urea, ammonia, uric acid and amino acid nitrogen. The other determinations were made as soon as possible.

Total nitrogen was determined by the official Kjeldahl method (Association of Official Agricultural Chemists, '25) and uric acid, amino acid, creatine and creatinine according to the methods described by Folin ('22). In the first study, urea and ammonia were determined by the colorimetric method and, in the second study, by the aeration method. All the data reported are averages of duplicate or triplicate determinations which checked within 2%.

<div style="text-align:center">RESULTS</div>

Table 1 gives a statistical interpretation of the daily variation in the 24-hour urinary excretion for each child when he received a constant medium protein diet. On account of the large volume of data, only the range in values and the mean with its probable error are given, together with the standard deviation and coefficient of variation.

In the second experiment, the lower range in urine volume as well as a reduction in the coefficients of variation from a high value of 11.2 to 8.9 indicates that the volume was more constant than in the first experiment. This is not surprising since the water intake was more carefully controlled during the second experiment. In spite of the changes in volume the specific gravity remained exceedingly constant, the coefficients of variation for five of the six children being 0.1.

Since the composition of identical diets varied somewhat from period to period and even between duplicate diets weighed on the same day (Hawks, Bray and Dye, '37), the

TABLE 1

Daily variation in 24-hour urinary excretion on a constant 3-gm. protein diet

FUNCTION	CHILD	EXPERIMENT	RANGE	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION
Volume (cc.)	D	I	820–1225	1034±17.0	115.6	% 11.2
	B		835–1330	1070±17.3	117.5	11.0
	J	II	585– 800	681± 9.6	60.4	8.9
	C		690– 970	800±10.3	68.6	8.6
	V		870–1130	998± 7.5	50.8	5.1
Specific gravity	D	I	1.010–1.015	1.0131±0.0002	0.0014	0.1
	B		1.012–1.017	1.0140±0.0002	0.0015	0.1
	J	II	1.008–1.013	1.0106±0.0002	0.0014	0.1
	C		1.008–1.013	1.0105±0.0003	0.0018	0.2
	V		1.007–1.012	1.0095±0.0002	0.0012	0.1
Total nitrogen (gm.)	D	I	7.09–7.80	7.46±0.03	0.22	2.9
	B		6.92–8.24	7.65±0.05	0.33	4.3
	J	II	4.53–5.18	4.93±0.03	0.17	3.4
	C		5.40–5.98	5.78±0.02	0.14	2.4
	V		6.31–6.96	6.71±0.03	0.19	2.8
Urea nitrogen (gm.)	D	I	6.30–6.99	6.65±0.03	0.21	3.2
	B		5.83–7.40	6.70±0.05	0.33	4.9
	J	II	3.91–4.53	4.31±0.02	0.15	3.5
	C		4.55–5.21	4.95±0.02	0.16	3.2
	V		5.40–6.04	5.75±0.02	0.17	2.9
Ammonia nitrogen (mg.)	D	I	185–279	226±3.8	25.5	11.3
	B		255–408	323±6.4	43.6	13.5
	J	II	92–125	110±1.6	10.0	9.1
	C		135–199	158±2.4	15.8	10.0
	V		141–194	168±2.5	16.7	10.0
Acidity (cc. 0.1 N acid)	D	I	170–229	199±2.4	16.2	8.2
	B		206–291	247±3.3	22.4	9.1
	J	II	63–108	85±2.3	14.6	17.2
	C		77–116	95±1.6	10.8	11.5
	V		83–140	112±2.1	14.4	12.8
Uric acid nitrogen (mg.)	D	I	75– 98	86±1.0	6.6	7.7
	B		94–122	110±1.0	7.1	6.5
	J	II	69– 77	73±0.4	2.5	3.4
	C		87–106	95±0.7	4.6	4.9
	V		97–113	107±0.6	4.3	4.1
Amino acid nitrogen (mg.)	D	I	49– 74	63±1.0	7.1	11.4
	B		56– 90	69±1.4	9.5	13.8
	J	II	54– 87	70±1.6	10.2	14.6
	C		68–109	87±1.9	12.6	14.4
	V		74–120	97±2.0	13.4	13.8
Creatinine nitrogen (mg.)	D	I	126–140	134±0.5	3.5	2.6
	B		131–148	141±0.7	4.8	3.4
	J	II	70– 78	75±0.4	2.3	3.1
	C		83– 91	87±0.4	2.6	3.0
	V		104–114	109±0.4	2.7	2.5
Creatine nitrogen (mg.)	D	I	90–118	108±1.0	6.5	6.1
	B		118–141	126±0.8	5.7	4.6
	J	II	74– 86	82±0.6	3.6	4.4
	C		75–100	92±1.1	7.0	7.6
	V		86–110	98±1.0	7.1	7.2

nitrogen intake for each child probably varied not only every period, but also on different days during a period. On account of these differences, one would expect the coefficients of variation for the urinary nitrogenous constituents to be at least as great as those found for intake in the two experiments which were 2.7 and 2.3. Table 1 shows that the coefficient of variation for total nitrogen for subject D was practically the same as that for the diet during the first experiment, while the coefficient for B was 4.3. In the second experiment, the values for J were less consistent than the figures for diet but those for the other two children varied to about the same extent. The increase in variability is in reality extremely small when one considers the number of uncontrollable biological errors, such as those connected with the collection of samples, the physical state of the child, exercise, and psychological reactions. Figure 1, which illustrates the daily fluctuations in the diet nitrogen in relation to the total urinary nitrogen excreted by each child, also indicates that the fluctuations for urinary nitrogen seem to be in somewhat the same proportion and not much greater than diet variations. Therefore, the data seem to indicate that the total urinary nitrogen excretion of the children had reached an equilibrium similar to or even equal to that for diet nitrogen.

The coefficients of variation for urea nitrogen, which were between 2.9 and 4.9, were slightly greater than those for total nitrogen. The increase, however, was probably insignificant. On the other hand, the values for ammonia nitrogen showed much greater variations, the coefficients ranging from 9.1 to 13.5. Since the urine specimens were kept on ice at all times and since the determinations were made as soon as the specimens were complete it scarcely seems possible that the increased variation was entirely due to the fluctuations in the decomposition of the urea although this may have been a determining factor. Since the values of ammonia varied to such an extent, it is not surprising that those for total titratable acidity varied in somewhat the same degree. The coefficients were lower in the first experiment, but higher in the second and ranged from 8.2 to 17.2.

DAILY VARIATION IN TOTAL URINARY NITROGEN AS RELATED TO A CONSTANT
AND ALSO TO AN INCREASE IN NITROGEN INTAKE

EXPERIMENT I   High Protein

Medium Protein
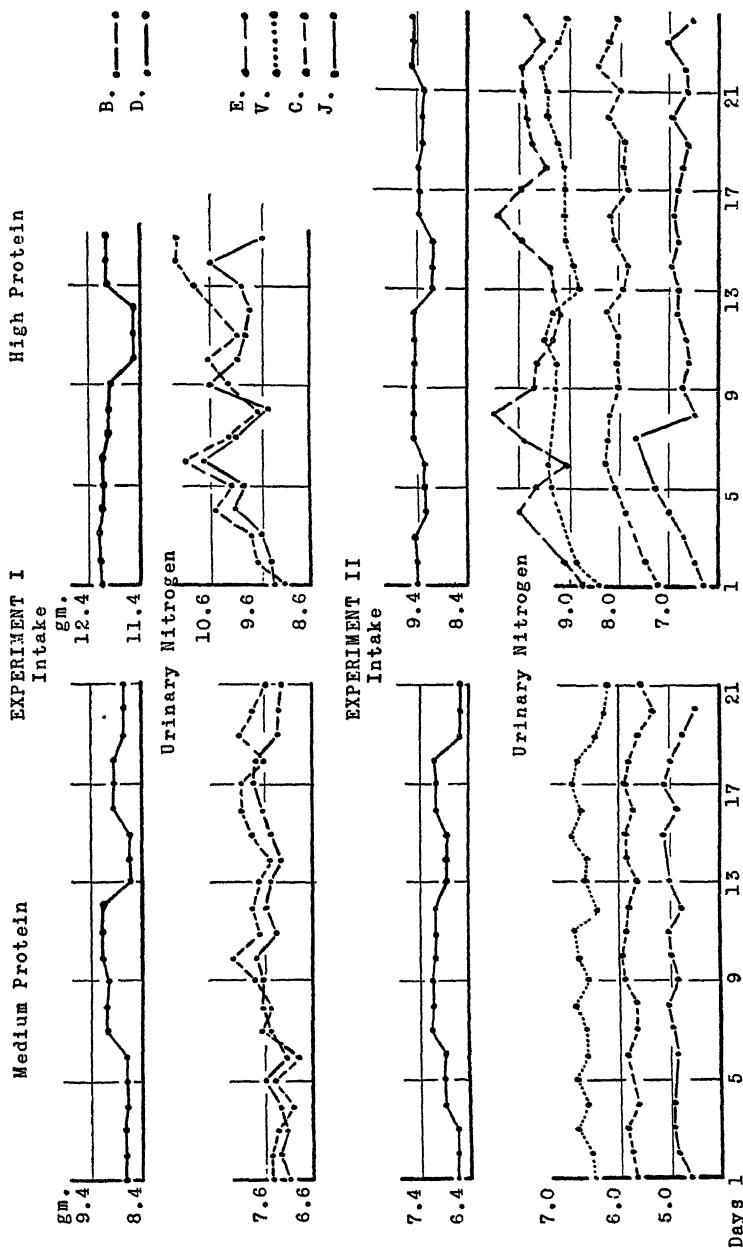
EXPERIMENT II

Figure 1

In the first experiment the coefficients of variation for uric acid nitrogen were about twice as high as those for total nitrogen, but in the second experiment they were only slightly increased. Since the composition of the diets in the two experiments was not identical, the changes in uric acid content of the diet may have caused the differences in variability. Although the diet in the first experiment contained about 6 gm. more of meat than that in the second, it does not seem that the uric acid from this source could have been twice as variable because the total diet nitrogen fluctuation was practically the same. Uric acid from vegetable or fruit source or from some metabolic factor must have caused the differences.

The values for amino acid nitrogen showed more irregularities than any of the other nitrogenous constituents. For the first experiment, the coefficients of variation were 11.4 and 13.8, practically the same as those for ammonia nitrogen, but for the second experiment they were slightly higher from 13.8 to 14.6. The values for each child seemed to fluctuate to the same extent.

Since creatinine is often considered to be an index of muscle metabolism and as a general rule remains fairly constant irrespective of diet, it is not surprising that the values should be as constant as those for total nitrogen. The coefficients of variation ranged from 2.5 to 3.4, and there was no more than 17 mg. of creatinine nitrogen difference between the highest and the lowest value for any one child. The creatine values, on the other hand, were considerably more variable, the coefficients of variation ranging from 4.4 to 7.6.

Pucher and his associates ('34) gave a statistical analysis of the variation in urinary nitrogenous constituents. Their results, however, cannot be compared directly with results in this study because their subjects were adults, the diet was not controlled except for the omission of meat on the days when samples were collected, and the collection days were not consecutive. The coefficients of variation which they report for total nitrogen, urea and creatinine are from five to six times greater than those reported here, but they find, as in this

study, that the values for these constituents are more constant than those for ammonia, acidity, amino acid and creatine. This fact may mean either that the technical errors in analysis of the latter constituents are greater than those for total nitrogen, urea, and creatinine or that some uncontrollable factor, not diet, influenced their excretion. The small variation in the uric acid excretion in this study may be due to the fact that the children consumed a constant amount of meat each day. If the variations which Pucher and his associates ('34) report may be considered normal for subjects living on an ordinary mixed diet, and if the coefficient of variation represented by the diet nitrogen may be considered as the minimum variation, the children in this study had certainly reached a fair degree of metabolic equilibrium, probably as great a degree as could be expected on account of the diet fluctuations.

Immediately following the change to a high protein diet, the majority of the urinary nitrogenous substances displayed more daily variation than they had on the medium protein diet. Figure 1 also shows the daily intake variation on the high protein diet as related to the total urinary nitrogen excretion of the six children. The intake values of the first experiment showed practically the same degree of irregularity as those on the medium protein diet, since the coefficient of variation was 2.3 as compared with 2.8. In the second experiment, the intake values were more constant on the high protein diet, the coefficient of variation being 1.6 instead of 3.0. Therefore, it is not surprising that the figures for total urinary nitrogen should seem to be more regular on the second than on the first experiment. Nevertheless, in all cases the total nitrogen increased rather regularly during the first 4 to 7 days. In some cases, the values became lower on the following 2 or 3 days and then they seemed to reach a more constant level. Since the figures for both intake and excretion fluctuated somewhat from day to day, it was impossible to say that the children reached an equilibrium at any one time. A 9-day period, however, apparently covered

the largest part of the irregularities and was thus considered the preliminary period.

Table 2 presents a statistical treatment of the data based on the entire 15 or 24 days of the high protein diet. It also gives the standard deviation and the coefficient of variation for the data during the first 9 days or the preliminary period for this diet, and in the second experiment, for the data on the last 15 days.

With the exception of creatinine all of the urinary nitrogenous substances increased in amount with the increase in diet nitrogen. The volume of urine remained practically the same and the daily variations were within a similar range. The figures for specific gravity averaged slightly higher, but the coefficients of variation were the same, 0.1. Total acidity increased in amount, but the coefficients of variation for each child were quite similar to those on the medium protein diet.

The change in the protein content of the diet, as indicated in figure 1, caused the total urinary nitrogen figures to be more irregular. The coefficients of variation, which ranged from 3.0 to 6.4, were slightly higher for each child, the older children having the highest values. The coefficient for E was slightly higher than the coefficients for the other children on the second experiment. His previous illness may have influenced the variability although 15 days had elapsed since he appeared normal. On the other hand, the larger fluctuation for the three older children may have been caused by their greater activity, or by a nervous tension which the younger children did not seem to have. The change in the nitrogen content of the diet must have been a factor because all other factors remained constant and the period by period variation in the diet was the same for subjects D and B and even lower for the other four children.

The alteration in the diet influenced some but not all of the other constituents. The coefficients of variation for each child for urea, ammonia and creatine were with few exceptions, higher than they had been on the medium protein diet. The values for uric acid and amino acid nitrogen showed no in-

Daily variation in 24-hour urinary excretion on a constant 4-gm. protein diet

FUNCTION	EXPERIMENT	CHILD	ENTIRE PERIOD				FIRST 9 DAYS		LAST 15 DAYS	
			Range	Mean	Standard deviation	Coefficient of variation	Standard deviation	Coefficient of variation	Standard deviation	Coefficient of variation
						%		%		%
Volume (cc.)	I	D	1009–1332	1156±14.3	82.3	7.1	69.7	5.9	...	...
		B	1063–1344	1187±15.4	85.5	7.2	96.4	8.0	...	...
	II	J	450– 770	670±11.1	78.9	11.8	60.2	8.6	85.6	13.1
		C	690– 900	813± 8.3	59.1	7.3	37.7	4.4	56.7	7.2
		V	730–1080	946±13.5	89.2	9.4	111.7	11.3	78.8	8.5
		E	730–1350	1046±24.6	171.3	16.4	164.5	14.4	153.4	15.5
Specific gravity	I	D	1.012–1.016	1.0142±0.0002	0.0012	0.1	0.0011	0.1	.....	...
		B	1.013–1.017	1.0146±0.0002	0.0012	0.1	0.0015	0.1	.....	...
	II	J	1.010–1.015	1.0129±0.0002	0.0013	0.1	0.0016	0.2	0.0011	0.1
		C	1.009–1.014	1.0122±0.0002	0.0013	0.1	0.0014	0.1	0.0008	0.1
		V	1.009–1.013	1.0115±0.0001	0.0009	0.1	0.0012	0.1	0.0007	0.1
		E	1.009–1.013	1.0113±0.0002	0.0011	0.1	0.0007	0.1	0.0013	0.1
Total nitrogen (gm.)	I	D	9.35–10.77	9.98±0.08	0.45	4.5	0.52	5.3	...	...
		B	9.15–11.33	10.35±0.12	0.66	6.4	0.57	5.6	...	...
	II	J	6.35– 7.65	6.84±0.04	0.27	4.0	0.43	6.3	0.15	2.1
		C	7.28– 8.46	8.04±0.04	0.26	3.3	0.38	4.8	0.17	2.1
		V	8.40– 9.61	9.21±0.04	0.28	3.0	0.43	4.8	0.21	2.2
		E	8.79–10.56	9.69±0.06	0.44	4.5	0.59	6.1	0.34	3.5
Urea nitrogen (gm.)	I	D	8.43– 9.82	8.99±0.07	0.42	4.7	0.51	5.7	...	...
		B	8.11–10.02	9.15±0.11	0.60	6.6	0.52	5.8	...	...
	II	J	5.52– 6.76	6.15±0.04	0.28	4.5	0.39	6.6	0.14	2.3
		C	6.44– 7.59	7.20±0.04	0.27	3.8	0.32	4.6	0.16	2.2
		V	7.71– 8.87	8.37±0.04	0.27	3.3	0.31	3.8	0.23	2.8
		E	7.91– 9.60	8.75±0.06	0.41	4.7	0.43	5.1	0.33	3.7
Ammonia nitrogen (mg.)	I	D	220–321	293±4.5	25.6	8.7	30.8	10.4	...	...
		B	360–527	428±8.8	48.7	11.4	56.9	12.9	...	...
	II	J	109–199	142±3.1	22.0	15.5	26.5	18.4	20.2	14.2
		C	185–279	217±3.5	24.6	11.3	30.3	13.8	22.2	10.2
		V	172–276	221±3.8	25.5	11.5	28.8	12.6	24.9	11.4
		E	171–285	233±4.6	31.9	13.7	35.8	15.4	30.8	13.2
Acidity (cc. 0.1 N acid)	I	D	202–265	236±3.2	18.2	7.7	19.3	8.0	...	...
		B	254–334	295±4.2	23.5	8.0	25.9	8.7	...	...
	II	J	76–123	94±1.6	11.5	12.2	11.2	12.1	12.0	12.7
		C	74–149	97±2.5	17.8	18.4	8.5	9.1	21.2	21.5
		V	90–139	115±2.2	14.5	12.7	15.3	13.0	14.7	12.9
		E	118–215	162±3.2	22.3	13.8	27.5	17.7	18.7	11.3
Uric acid nitrogen (mg.)	I	D	97–113	105±0.8	4.5	4.3	4.5	4.3	...	...
		B	119–161	138±2.0	11.2	8.1	8.6	6.5	...	...
	II	J	73– 84	79±0.4	2.8	3.6	2.9	3.8	2.7	3.4
		C	95–111	102±0.6	4.4	4.3	2.0	2.0	3.9	3.7
		V	108–125	116±0.7	4.9	4.3	6.7	5.9	4.1	3.5
		E	92–109	100±0.7	4.6	4.7	3.9	4.1	4.0	3.9
Amino acid nitrogen (mg.)	I	D	80–100	90±1.1	6.1	6.8	4.9	5.7	...	...
		B	83–120	96±1.9	10.4	10.8	5.2	5.7	...	...
	II	J	99–160	139±2.6	18.5	13.3	18.4	15.3	7.0	4.7
		C	123–204	177±3.4	24.4	13.8	20.2	13.6	8.7	4.6
		V	132–222	193±3.9	25.9	13.4	30.3	18.8	11.8	5.8
		E	148–257	207±4.4	30.6	14.8	21.6	12.4	15.9	7.1
Creatinine nitrogen (mg.)	I	D	133–166	150±2.2	12.5	8.3	10.2	6.5	...	...
		B	148–170	156±1.2	6.5	4.2	6.6	4.2	...	...
	II	J	71– 78	74±0.2	1.7	2.3	1.6	2.1	1.7	2.3
		C	82– 90	86±0.3	2.4	2.8	2.1	2.4	2.6	3.1
		V	100–112	106±0.6	3.8	3.6	1.7	1.5	2.5	2.4
		E	118–126	122±0.3	2.4	2.0	2.9	2.4	2.2	1.8
Creatine nitrogen (mg.)	I	D	130–189	155±2.8	15.4	10.0	20.4	13.1	...	...
		B	160–211	188±2.5	14.1	7.5	14.1	7.7	...	...
	II	J	74–102	90±1.2	8.8	9.9	6.2	6.6	9.5	10.8
		C	78–108	92±1.1	8.1	8.7	9.3	9.6	6.5	7.2
		V	97–122	108±1.0	6.5	6.0	9.2	8.2	4.9	4.6
		E	100–152	121±2.2	15.6	12.9	14.7	10.8	7.8	7.0

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crease in variability and those for creatinine remained just as constant as they had been on the medium protein diet.

During the first 9 days of the high protein diet, those constituents which were apparently most influenced by diet, usually showed a greater irregularity than they did for the entire period. The coefficients of variation for total nitrogen, urea and ammonia were higher for every child, except B, and those for creatine increased for four of the six children. The other constituents showed no constant change.

In the second experiment, there was less variation for some of the constituents during the last 15 days of the high protein diet than there had been at any time during the entire study. The coefficients of variation for total nitrogen and urea were lower than they had been on the medium protein diet. The values for ammonia, acidity and uric acid were no more constant than they were when all of the data on the high protein diet were considered. The figures for amino acid nitrogen were more constant than they had been at any other time. With the exception of the figures for J, the creatine values were less variable than they had been for the total high protein diet, but not as constant as they were on the medium protein diet. Creatinine figures seemed to be exceedingly constant throughout the entire study and were apparently affected little by the change in diet. The variability in the values for the two children in the first experiment and for V was slightly greater on the medium protein diet.

The data for total nitrogen on the last 15 days of the high protein diet were just as constant as that on the medium protein diet. Thus it can be assumed that, if the children were in nitrogen equilibrium during the medium protein diet, they were probably in equilibrium during the last 15 days of the high protein diet and that the 9-day preliminary period was sufficiently long for this study. Greater changes in diet might require a longer preliminary period.

The individual differences among the children were in reality quite small. In general, the coefficient of variation for one particular constituent was almost the same for each

child.   The increase in variability following the change in diet was again similar.   Therefore, the data seem to indicate that under identical conditions the children reacted the same.

So far the discussion has dealt entirely with the variability of the data obtained on individual children and not with the variability in the data as a whole.   In order to combine the figures for several children it was necessary to use the values expressed in terms of a common measurement, milligrams per kilogram of body weight.   Table 3 gives the coefficients of variation obtained from the statistical treatment of the data per kilogram on each experiment and for both experiments together, for the medium and high protein diets and also for the first 9 days of the high protein diet, and for the last 15 days of the high protein diet on the second experiment.

The combination of the intake values per kilogram for several children produced coefficients of variation which were somewhat different from those for the data of individual children.   For the first experiment the values were similar, but for the second and for both experiments considered together the coefficients were increased.   In all cases the figures were slightly more variable on the medium than on the high protein diet.   It is probable that several factors beside the variation in the composition of identical diets produced this variability, because, even though the children received food in amounts proportional to their body weight. the amount of nitrogen consumed varied slightly from child to child.   In the first place, it was impossible to get an exact proportion of each food without using small fractions of a gram which would have been impractical.   Secondly, the weights of the children changed somewhat during the experiment.   Finally, there were greater differences between the nitrogen intakes of the children on the two experiments than there were between different children on the same experiment.

In general, the coefficients of variation for all of the nitrogenous excretory products were higher than they were for individual children.   Nevertheless, the data for total nitrogen and urea on each experiment showed the same tendencies as

they did for individual children. In spite of the lower varia-
tion in intake on the high protein diet, the coefficients of
variation increased following the change in diet. The co-
efficients were highest during the first 9 days on the high
protein diet and similar to those on the medium protein diet
on the last 15 days of the experiment.

TABLE 3

Coefficients of variation of the per kilogram intake and urinary output

FUNCTION	EXPERIMENT	MEDIUM PROTEIN DIET	HIGH PROTEIN DIET		
			All	First 9 days	Last 15 days
Intake	I	% 2.3	% 2.0	% 1.0	%
	II	3.8	3.2	3.0	3.3
	Both	6.4	5.5	5.8	
Total urinary nitrogen	I	4.7	5.9	6.5	
	II	4.8	5.2	6.0	4.8
	Both	6.1	5.9	6.6	
Urea nitrogen	I	5.6	6.5	7.4	
	II	4.3	5.5	6.0	4.7
	Both	7.1	6.1	7.2	
Ammonia nitrogen	I	18.7	18.2	20.0	
	II	15.1	17.9	19.4	17.3
	Both	28.5	27.7	30.4	
Uric acid nitrogen	I	10.3	11.4	9.7	
	II	9.1	11.5	11.3	11.3
	Both	11.3	11.7	11.1	
Amino acid nitrogen	I	12.3	8.8	6.1	
	II	15.3	15.0	14.8	8.7
	Both	25.5	31.1	32.5	
Creatinine nitrogen	I	3.4	7.1	6.7	
	II	7.1	8.6	8.6	8.5
	Both	12.9	17.7	19.4	
Creatine nitrogen	I	6.1	10.0	10.7	
	II	7.7	10.0	10.0	7.7
	Both	7.7	20.5	18.4	
Acidity	I	10.9	10.5	10.4	
	II	13.7	19.3	18.1	20.0
	Both	34.6	38.2	39.5	
Volume	I	11.3	7.7	7.7	
	II	9.7	13.3	12.7	12.5
	Both	10.8	14.2	12.6	

The collective data for total nitrogen and urea in the two experiments showed the highest coefficients of variation on the first 9 days of the high protein diet, but the variation in all of the data for the high protein was less than that for the medium protein diet. Nevertheless, a comparison of the co-efficients of variation for intake and total nitrogen showed that the values on the medium protein diet decreased from 6.4 to 6.1 and those on the high protein diet increased from 5.5 to 5.9. Therefore, the collective values probably show the same tendencies as do the individual figures, but the differences between the children and between the two experiments partially mask those tendencies.

The data for ammonia and creatine are less conclusive. In the second experiment the figures display the same tendencies as total nitrogen, but on the other experiment and on the collective data the relationship was not clearly portrayed.

The values for uric acid, amino acid, acidity and volume showed approximately the same variability on both diets. Thus the collective figures, as well as those for individual children, indicated that these constituents were not influenced by diet. The figures for creatinine became more variable after the change to the high protein diet, but they were not more irregular on the first 9 days. Thus, as indicated by the data on individual children, the diet had less influence on creatinine than on total nitrogen.

The results from the collective data indicate that individual tendencies may be obviated by combining the data because the collective data show more variation than that for one experiment or that for an individual child. The collective data also point out the danger of combining figures from experiments which were not identical. Although the two experiments in this study were practically the same, the differences were great enough to influence the variability of the results.

SUMMARY

1. The amount of total urinary nitrogen, urea, creatinine and, in one experiment, uric acid excreted by six children on constant medium protein diets following a 10- or 12-day preliminary period varied to approximately the same degree as the values for diet nitrogen.

2. Uric acid in one experiment and creatine in both were about twice as variable as diet nitrogen, while ammonia, acidity and amino acid showed more irregularity.

3. The increase in the protein content of the diet caused the values for total nitrogen, urea, ammonia and creatine to be more variable, especially during the first 9 days. Then the figures reached an equilibrium similar to that on the first diet. Therefore, in this study a 9-day preliminary period seemed to be adequate.

4. The change in diet did not seem to influence the variability of the data for acidity, uric acid, amino acid or creatinine.

5. Individual children tended to react in a similar manner both to the constant diet and to the change in the protein content of the diet.

6. When the values were expressed on the basis of kilograms of body weight, the coefficients of variation of the data became larger in all cases. The values for either experiment showed the same tendencies as the data for individuals, but the collective values for the two experiments tended to obscure the fluctuations.

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A RESPIRATION CHAMBER FOR USE WITH HUMAN SUBJECTS [1]

L. H. NEWBURGH, MARGARET W. JOHNSTON, F. H. WILEY,
J. M. SHELDON AND W. A. MURRILL

Department of Internal Medicine, Medical School, University of Michigan

THREE FIGURES

The respiration chamber devised by F. G. Benedict et al. ('34) for large animals has been modified by us for the study of the human subject. It was constructed to measure the total transformation of energy (heat production) during 24 hours. It may also be used for longer or shorter periods. The shortness of the period is necessarily determined by the time required for complete mixing of the gases. For example, about 80 liters of CO_2 were rapidly introduced into the closed chamber in which the mixing pump was running. The inlet was then stoppered. Samples withdrawn 30 minutes later gave a value of 0.816% CO_2. After another 30 minutes, samples yielded a value of 0.815%. Therefore complete mixing had occurred by the end of the first ½ hour. The apparatus employs the principle of open circuit indirect calorimetry.[2]

The subject is enclosed in a sealed space from which air is removed at a constant rate by means of a pump. Spirometers continuously sample the air leaving the chamber so that they contain a true aliquot of all the outgoing air. This aliquot is analyzed for its percentile composition of carbon dioxide and oxygen. We employ the apparatus devised for this pur-

[2] An extended discussion of this method has been presented by E. F. DuBois ('36).

pose by T. M. Carpenter ('33). As pointed out by Carpenter we were able to make duplicate analyses which checked within 0.004% for CO_2 and 0.005% for O_2. The apparatus was frequently checked by analyses of outdoor air. The total volume of outgoing air is recorded by calibrated gas meters. The incoming air is outdoor air which has a fixed percentile composition of carbon dioxide and oxygen. When the composition and the volume of the outgoing air and the composition of the incoming air are known, one may calculate the absorption of oxygen and production of carbon dioxide. To calculate the heat production one must also determine the urinary nitrogen. With these data, the calculation proceeds in the standard manner.

The chamber (fig. 1) consists of an inverted tank or hood hung on four steel cables that pass over pulleys near the ceiling of the laboratory to counterpoises whose combined weight is roughly equal to that of the hood. The upper and lower corners of the hood are supplied with rollers that fit into channels which are fastened to the floor and ceiling of the laboratory. These channels fix the position of the hood and guide its upward and downward movement. When the hood is pulled down the lowest 2 inches of its walls descend into oil[3] contained in a trough which is sealed into the concrete floor.

The spirometers (fig. 2) are of the type used to measure vital capacity.[4] However, the tube through which the gas passes into the sealed space is only $\frac{1}{16}$ inch inside diameter. The volume of the bell is about 7 liters. The counterpoise is specially designed to move in the sleeve with the least possible friction, and carries a pointer that moves past a graduated bar. The bell is attached to the weight by a chain that passes over a pulley whose axle and the seat in which the axle rides are carefully milled to reduce friction. Oil[3] is used as a seal. Since the weight of the bell will increase relative to the weight of the counterpoise as the former moves up out of the oil,

[3] Squibb's mineral oil.
[4] Obtained from Warren E. Collins, Inc., 555 Huntington Ave., Boston.
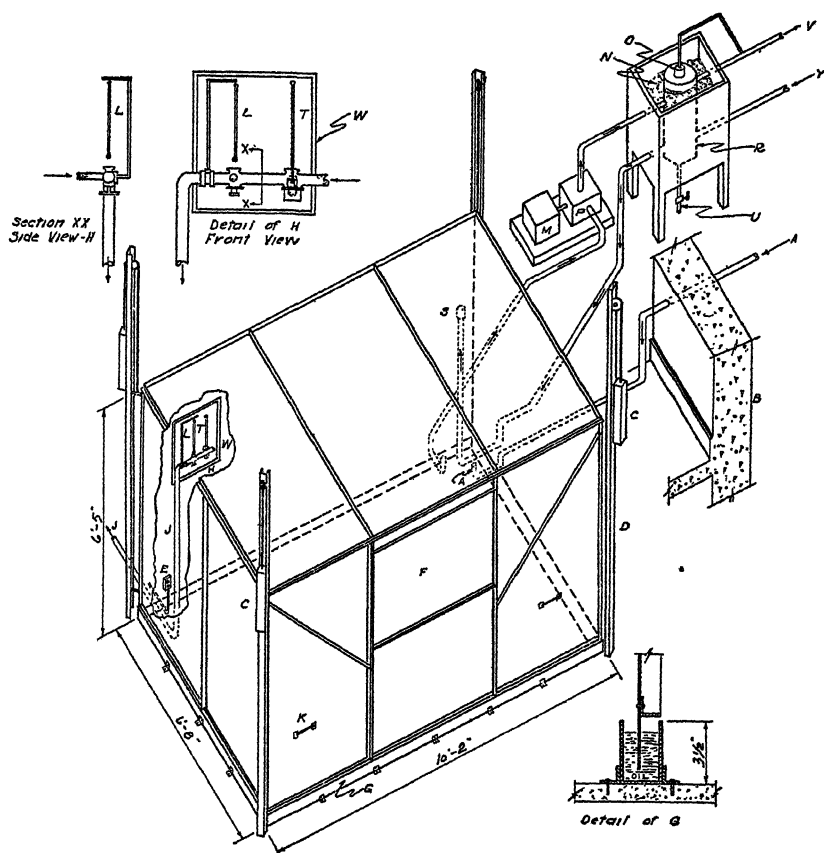
Fig. 1 Diagram of the chamber. A, duct through which outdoor air enters the chamber. B, outer wall of laboratory. C, lead weights, attached to the upper corners of the chamber by means of flexible steel cables that pass over pulleys in the top of the channels, D. The upper and lower corners of the chamber are supplied with rollers that move in the channels. D, the channels are fastened to the floor and ceiling of the laboratory. E, electric outlet. F, plate glass, sealed into wall of chamber. G, steel trough containing mineral oil. T, wet bulb thermometer in the upper end of the duct, J, through which the air leaves the chamber. H, the wick of the wet bulb thermometer dips into a wide mouthed bottle containing distilled water and held in position by rubber bands. The position of the valve causes the air to pass by the wet bulb or the wet bulb may be excluded from the circuit. A loose ball of copper wire soaked in oil, in each of the inlets to duct J, filters the air. K, handles.

The assembly whose parts are marked with the letters M through Y is a device for lowering humidity and cooling the air within the chamber. M and P, motor and pump. Cold brine enters a coil suspended inside of the sheet brass cylinder, R, and leaves through V. The rate of flow of the brine is regulated through the thermostatic valve, O. The cylinder is embedded in granulated cork, N. Water that condenses in the cylinder is removed at the end of an experiment by means of valve U. W, window in wall of chamber to permit observation of the wet bulb thermometer, T, and the dry bulb thermometer, L.

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special provision must be made to insure uniform rate of movement of the bell throughout its course. This is accomplished by using a counterpoise of proper weight and a chain of proper weight and length.

After passing the spirometers, the pipe that carries the main stream of outgoing air approaches the gas meters. They are of the type commonly employed to record the flow of illuminating gas into a building. The hands on the last meter move at one-tenth the rate of those on the first and second meters. They were calibrated, after the whole apparatus had
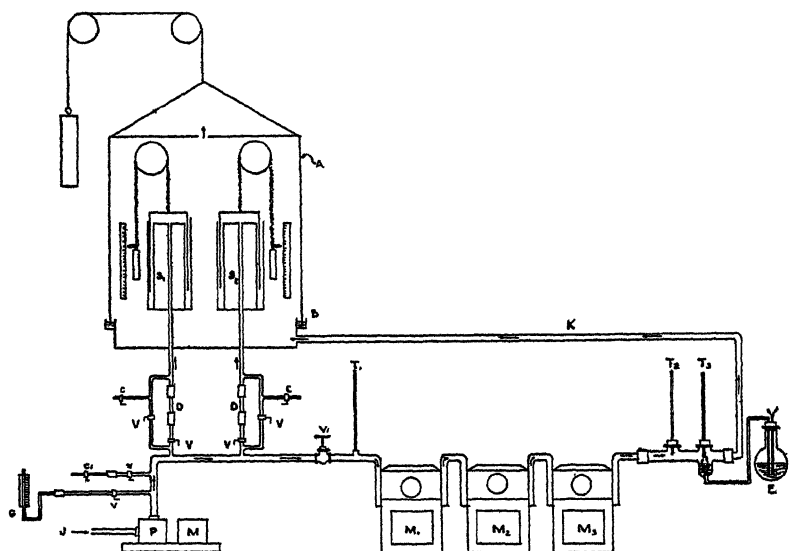
Fig. 2 Spirometers and gas meters. A, hood which when closed causes the spirometers to be surrounded by chamber air. A hole in the top of the hood allows escape of air. B, trough for water seal. S_1, S_2, spirometers whose bells are hung on chains that pass over pulleys to counterpoises. C, thick walled rubber tubing and clamp, through which samples from the spirometers are withdrawn. C_1, similar device for obtaining samples of outgoing air. D, glass capillary tubes. The rate of sampling is regulated by the internal diameter of these tubes. V, one-way brass valves. V_1, globe valve. G, mercury manometer. J, continuation of duct through which the air leaves the chamber. P, pump driven by electric motor M. M_1, M_2, M_3, gas meters. T_1 and T_2, dry bulb and T_3, wet bulb, thermometers. E, flask containing distilled water. K, duct that leads the outgoing air into the box that encloses the spirometers.

been set up by connecting a large cylinder of nitrogen weighed to 0.1 gm., to a Douglas bag and then to the outgoing pipe of the chamber. After the bag had been partly inflated the meters were read and the pump turned on. As the bag emptied more gas was allowed to flow into it from the cylinder until the latter was empty. The instant the bag had emptied, the pump was stopped and the meters read again. Factors for each meter were obtained in the usual manner.

Beyond the last meter, the stream of air passes across dry and wet bulb thermometers and thence into the bottom of the compartment that contains the spirometers. This compartment consists of two sections. The lower one is a shallow box carrying a trough on the outer face of its walls. The trough contains a water seal. The upper section is a hood that encloses the spirometers. Its lower edges fit into the water seal. The outgoing air is led back into the compartment in order to immerse the spirometers in a gas mixture whose concentration of carbon dioxide is similar to that within the spirometers. The air escapes through an opening in the top of the compartment. The purpose of this arrangement is to prevent diffusion of carbon dioxide from the spirometers. Without this precaution, we were first able to detect diffusion when the concentration of carbon dioxide had reached 0.45%.

Diffusion from the chamber itself must also be considered. In order to deal with this question we allowed CO_2 to flow into the chamber through the sampling duct and then closed the inlet pipe. The gas within the chamber was mixed as usual. When the concentration of CO_2 within the space was 0.475%, no change was detectable 20 hours later. However, when the initial concentration was 0.616%, it fell to 0.606% in 21 hours. An initial concentration of 0.816% was reduced to 0.801% in 22 hours. Expressed in volumes, these data show that 1 liter of CO_2 was lost when the initial concentration was 0.616%; and that 1.5 liters of CO_2 were lost when the initial concentration was 0.816%. Accordingly it is advisable to prevent the concentration of CO_2 in the chamber from rising above 0.5% by proper regulation of the ventilation.

However, when the concentration of CO_2 is 0.6% for not more than 4 hours, the concentration will fall only 0.002%—a negligible change.

In order to make the chamber independent of the weather, the air is cooled and dried by refrigeration. A ½ inch pipe is brought out of the chamber without breaking the seal and is attached to an air pump. The latter delivers the air into the top of a closed sheet copper cylinder. Several inches above the bottom of the copper box a second pipe leads the air back into the respiration chamber. Running through the copper container is a cold brine line supplied with fins. The rate of flow of the brine is controlled by an automatic valve activated by the temperature of the air. The bottom of the copper vessel is furnished with a valve by means of which water may be drained away at the end of an experiment.

The chamber contains a standard single bed, a chair, a table, a reading lamp, telephone and radio connection. The subject is supplied with food of the desired composition contained in air tight vessels. The urine is voided into enameled cans whose lids are designed to minimize evaporation. The stool is passed into large tin cans (diameter 8 inches) whose lids fit so tightly that the annoyance of prolonged fecal odor within the chamber is done away with. A commode is on hand for subjects who desire to use it.

THE VOLUME OF THE CHAMBER

The inside dimensions of the closed chamber were measured and the volume calculated. They are: length, 3.043 M; width, 1.992 M and height, 1.922 M. The calculated volume is 11,650 liters. This volume is decreased somewhat by the necessary furnishings of the chamber and by the subject who is in it. On the other hand, it is augmented by the pipes leaving the chamber, the blower and the meters. Consequently we have used the value of 11,600 liters as the working volume. This must be close to the true volume and if it were wrong by 100 liters the error involved in the calculation of the respiratory exchange would be less than the error of the whole method.
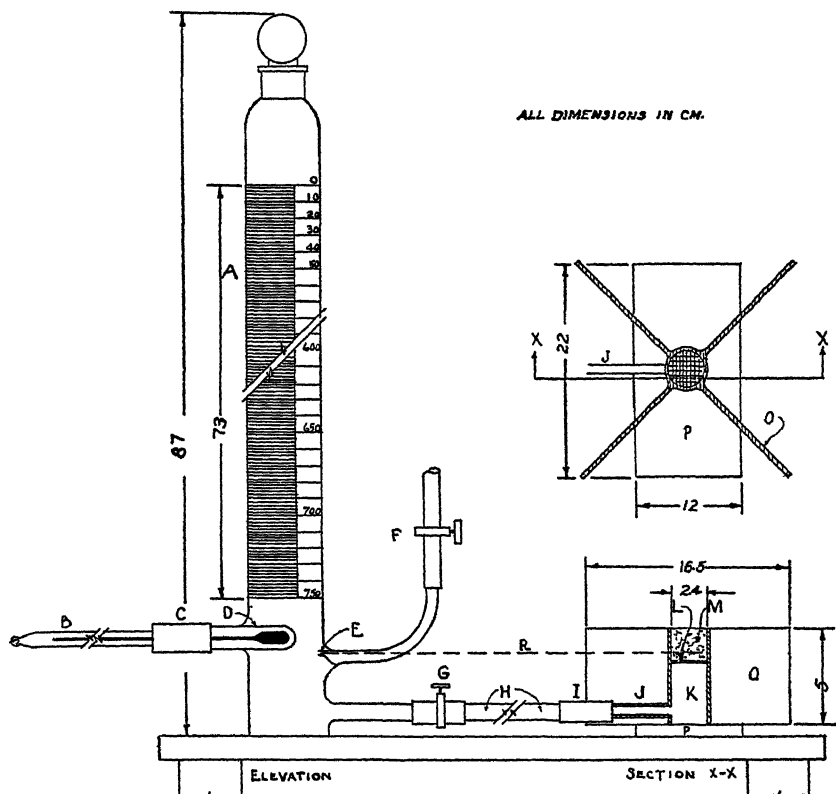
Fig. 3 Alcohol burner. A, cylinder graduated to 2 mls. and closed by a ground glass stopper (air tight). B, thermometer held in thermometer seal, D, by the rubber tubing, C. E, capillary inlet for air. The alcohol in the burner seeks the level of the air inlet. This capillary inlet keeps the bubbles small so that there is no interference with the volume readings. Openings, E and H, are closed by clamps, F and G, while filling cylinder. The outflow tube, H, connects cylinder with burner by rubber tubing, I, to copper tube, J. K, chamber in which alcohol collects and rises to level, R. L, screen on which shredded asbestos, M, rests. O, copper fins to carry away the heat of the flame. P, base of burner.

To regulate the flame, the apparatus is set up so that the level of alcohol in the burner is between the screen and the top of the burner. Shredded asbestos, the highest part of which is above the original level of the alcohol, is placed loosely in the burner. It is then lighted and when the alcohol burns evenly, the level of alcohol in the feeding cylinder, A, and the time are recorded. Then after a period of 10 minutes, for example, another reading is taken and the milliliters per hour calculated. By changing the level of the asbestos the flame can be adjusted to consume alcohol to within 1 or 2 ml. per hour of the desired rate.

For example, a difference in the concentration of 0.1% in carbon dioxide content of the chamber between the beginning of the experiment and the end would result in an error of only 0.1 liter CO_2 for every 100-liter error in volume of the chamber. This is a change in concentration of carbon dioxide considerably greater than that obtained in the usual type of experiment.

ALCOHOL CHECKS

The whole apparatus is checked for tightness, recovery of oxygen and carbon dioxide by means of alcohol checks. An alcohol lamp (fig. 3) especially designed for the chamber is used. The standard procedure for the study of human subjects is used. The lighted lamp in which the rate of burning is adjusted carefully, is placed in the chamber at night; the chamber closed and ventilation started. The following morning samples of air are removed from the chamber for analysis, the volume of alcohol is read on the graduated cylinder, the meters read and the collection of the aliquot samples in the spirometers begun. The experiment continues for 24 hours when the chamber is again sampled, the spirometers closed and the meters and alcohol cylinder read, to end the experiment. The data obtained permit the calculation of the respiratory quotient, the oxygen absorption and the carbon dioxide production.

A series of checks is shown in table 1.

TABLE 1
Alcohol checks

DATE	ALCOHOL BURNED	R.Q.	CARBON DIOXIDE			OXYGEN		
			Theoretical	Determined	Recovery	Theoretical	Determined	Recovery
	gm.		*liters*	*liters*	*%*	*liters*	*liters*	*%*
7- 5–35	260.9	0.668	254.0	253.7	99.9	281.1	279.9	99.7
9–17–35	369.0	0.682	359.4	357.1	99.4	539.2	523.8	97.1
9–30–35	428.3	0.662	417.0	412.6	98.9	625.5	623.0	99.6
4- 8–36	297.3	0.661	289.1	287.5	99.4	433.7	434.3	100.1
6–25–36	335.3	0.660	326.4	319.3	97.8	489.7	483.5	98.7
Average		0.667			99.1			99.0

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FURTHER EXPERIENCES WITH THE MEASURE-
MENT OF HEAT PRODUCTION FROM
INSENSIBLE LOSS OF WEIGHT [1]

L. H. NEWBURGH, M. W. JOHNSTON, F. H. LASHMET AND
J. M. SHELDON

Department of Internal Medicine, Medical School, University of Michigan

It is well known that vaporization of water is one of the mechanisms by means of which the body rids itself of heat. This portion of the outgoing heat is removed at the rate of 0.58 of a large calorie per gram of water vapor.

It has been suggested that a quantitative relationship exists between the total heat elimination and that portion which is removed by the vaporization of water. We have been studying this relationship and now propose to deal specifically with the following questions: 1) Can an accurate procedure be devised for the calculation of water vapor? 2) Does the organism always lose a fixed percentage of the heat of its metabolism by vaporization of water, and if not, are there conditions when it will do so?

The direct measurement of water vapor is very difficult. However, since the water vapor always accounts for the greater part or all of the insensible loss of weight, the possibility of calculating it from the latter exists.

The insensible loss of weight is easily obtained by substitution in the following equation:

I.L. = (Initial Body Weight + Ingesta)—(Final Body Weight + Urine + Stool),

where I.L. is insensible loss of weight.

The composition of the insensible loss of weight is accurately defined in the following equation:

$$\text{I.L.} = \text{I.W.} + CO_2 - O_2,$$

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where I.W. is the weight of the water vapor or insensible water; and CO_2 and O_2 are the weights of the two respiratory gases. It must be understood that insensible water means only that water which leaves the body in the form of vapor. The water vapor is always the largest part of the insensible loss of weight and may be the whole of it, as already noted. When the organism is metabolizing a mixture that necessitates an absorption of oxygen whose weight equals that of the carbon dioxide produced,

$$I.W. = I.L.$$

This same fact may be expressed in terms of the respiratory quotient. Thus when the weights of the two gases are equal, their volume relationships are such that the respiratory quotient is 0.725. As is generally understood, such a metabolic mixture is one in which a very small proportion of the calories are yielded by the oxidation of carbohydrate. As the carbohydrate of the metabolic mixture increases, the weight of the carbon dioxide becomes proportionately greater than that of the oxygen. When the mixture, for example, is such that the respiratory quotient is 0.90, the organism is producing 0.9 liter of carbon dioxide for every liter of oxygen it absorbs. The corresponding weights of the two gases are then 1.769 gm. and 1.429 gm., and the difference is 0.34 gm. for every liter of oxygen absorbed.

In order to derive the weight of the water vapor from the insensible loss of weight one must, therefore, determine the weights of the carbon dioxide and oxygen for the period.

We have already published several papers dealing with these questions. In the first one (Wiley and Newburgh, '31) it was shown that a naked subject in the post-absorptive state, lying quietly upon steel ribbons, lost a different percentage of the heat of his metabolism through vaporization of water with every change in the temperature of the air surrounding him. When, however, he clothed himself in such a way that he felt neither uncomfortably cool nor warm, he exhibited a striking tendency to lose a fixed percentage of the heat by

evaporation of water, over a considerable range of environmental temperatures.

Subsequently, we determined what percentage of the total heat was removed by vaporization during consecutive 24-hour periods by a group of men and women who continued to lead their usual lives (Newburgh, Wiley and Lashmet, '31). They were asked to adjust their clothing to avoid conscious discomfort from heat and cold. The experiments were conducted during the winter months. The subjects spent most of their waking hours in buildings heated to the usual temperature, and each of them was out-of-doors several times each day for a few minutes at a time. They all slept with the windows of the bedroom open. They were asked to avoid strenuous exercise such as hand ball. Two of the subjects were diabetics. The remainder of the group were normal young men and women busily occupied with the care of patients or with work in the laboratory. The nude, fasting weight of each individual was recorded each morning at the same time on a balance[2] sensitive to 1 gm. The prescribed diet was prepared and weighed in the special diet kitchen of the hospital. The foods used varied from day to day. The weights of the urine and stools were determined each 24 hours.

The study was based on the following conceptions: A well-nourished adult who is following a routine of life and who is fed a fixed diet, the calorific value of which approaches his maintenance requirement, will shortly establish nitrogen balance. In addition, the glycogen store in the liver will come into balance with the carbohydrate of the diet. Thereafter, the individual will oxidize carbohydrate in the amount supplied by the diet provided the time interval under consideration is not too brief. It was not expected that this principle would hold for each cycle of 24 hours, but that the subject would be in carbohydrate balance if each period lasted 1 week. Under these circumstances, change in body weight could be attributed to deposition or loss of either adipose tissue or water. Since there was no reason for suspecting a disturbance of water metabolism in regard to any of the individuals,

[2] Obtained from Henry Troemner, Philadelphia.

change in body weight was attributable to adipose tissue provided the observations of any subject were continued for several weeks. When the record was completed, the average daily metabolic mixture corresponded with the diet except that any change in body weight over the whole period had to be reduced to a daily average, and then 90% of it either added to or subtracted from the dietary fat.[3] The calories of this metabolic mixture were taken to be the daily heat production of the individual The total change of weight of three of the subjects (table 1, T.M., M.W., M.P.) was insignificant. In those instances, heat production equalled the calories of the diet.

Having arrived at the metabolic mixture, the production of carbon dioxide and the absorption of oxygen could be calculated. Since the insensible loss of weight had been determined for each 24 hours, the weight of the insensible water could be derived by means of the equation

$$I.W. = I.L. - (CO_2 - O_2).$$

When I.W. is known, the heat removed by it is simply I.W. $\times$ 0.58. From this value and the heat production, the percentage of heat removed by the vaporization of water was calculated by means of the expression

$$\frac{I.W. \times 0.58}{Heat\ Production}$$

In order to arrive at a diet that would suit our purpose we fed what experience led us to believe was a maintenance diet, measured the insensible loss of weight daily for at least 1 week, calculated the gaseous exchange for that diet, derived the I.W. and assumed that 24% of the heat was removed by vaporization. The final diet was then constructed to contain the number of calories indicated by this approximation to the individual's heat production. The distribution of the various foodstuffs was the usual one for this part of the world.

[3] Since adipose tissue contains 10% of water and dietary fat is expressed in anhydrous terms, 90% of the change in weight was used.

However, in the case of the two diabetic subjects, it was not necessary to use a circuitous method to arrive at the materials oxidized since both had been living on carefully prescribed, weighed diets for years; and had long since come into balance.

In two instances, the available energy of the diet was determined by combusting a sample in the bomb calorimeter; and by subtracting from the value thus obtained, the residual heat in the urine and feces, also measured by means of the

TABLE 1

Average percentile loss of heat by vaporization of water

SUBJECT	PERIOD	AGE	SEX	HEAT LOST BY VAPORIZATION OF WATER	REMARKS
	days	*years*		*%*	
First group					
T.M.	72	56	M.	23.8	Diabetic
F.D.J.	5	30	M.	24.2	Diabetic
F.H.W.	18	28	M.	24.2	Chemist
M.W.	44	24	F.	24.4	Chemist
M.P.	35	25	M.	24.1	Medical student
A.W.	68	23	M.	24.7	Graduate student
R.L.G.	72	24	M.	25.2	Graduate student
Second group					
B.DeV.	145	24	M.	24.2	Student
H.J.	30	18	M.	24.3	Patient in bed
S.	30	15	M.	24.8	Patient in bed
P.	30	18	M.	24.8	Patient in bed
W.B.	25	47	M.	24.7	Patient in bed

calorimeter. These checks were in good agreement with the calorific values of the diets calculated from the food tables.

Table 1 shows the average percentage of the total heat lost through evaporation of water by each subject; but it gives no information about the extent of daily variation from the average.

This first group of seven adult persons whose lives were restricted only in the few important ways noted above, showed a striking tendency to rid themselves of the same proportion of the heat of their metabolism by vaporization of water.

The average value for the group was 24.4%. The individual variation was from 23.8% to 25.2%.

Subsequently a second group of individuals was studied (table 1). To obtain greater accuracy, the nitrogen exchange was actually determined and the diets were restricted to a few foods whose composition showed little tendency to vary. They were milk, 40% cream, butter, bread, cheese, eggs, jelly and canned grapefruit. The cheese was obtained in 5-pound bricks and samples were analyzed for nitrogen and water at the beginning and end. The diets were prepared in our laboratory and all the weighings were made by the investigators.

The data from one of this second group of subjects are reported in some detail.

B. DeV. was a male, 24 years of age, 5 feet, 11 inches tall. He was a student in the school of architecture. He occupied a room set aside for the purpose in the laboratory. He walked 4 to 6 miles each day in the open to classes. He ate the diet for 3 weeks before the study began.

	gm.
Body weight October 9, 1933	60,108
Body weight March 3, 1934	60,900
Total gain	792
Gain per day	5.46
Dietary nitrogen, average per day	9.01
Excretory nitrogen, average per day	8.91
Balance	+0.10

Since he was in nitrogen balance, the added body weight was assumed to be adipose tissue and since approximately 10% of the latter is water, it was assumed that he stored 4.91 gm. of fat daily containing 45.7 Calories (4.91 × 9.3).

He ingested 2750 Calories and stored 45.7 Calories, therefore, he produced 2704 Calories. Since the diet was eaten just after being taken from a refrigerator it was cold. We calculated that it required 40 Calories to bring it up to body temperature. The dissipation of heat was accordingly 2664 Calories (2704 — 40). The diet had the following composition: 9.01 gm. nitrogen; 156 gm. fat; 279 gm. carbohydrate.

Since he was in nitrogen and carbohydrate balance and had stored 4.9 gm. fat daily, the metabolic mixture was 9.01 gm. nitrogen, 151 gm. fat and 279 gm. carbohydrate. The difference between the weights of the outgoing carbon dioxide and incoming oxygen for this metabolic mixture may now be calculated by means of the factors: grams urinary $N \times 0.84$; grams carbohydrate oxidized $\times 0.41$; grams fat oxidized $\times -0.08$. This gives 110 gm. $(7.57 + 114.39 - 12.08)$, for $CO_2 - O_2$. The 24-hour insensible loss of weight was 1220 gm. and the insensible water was therefore 1110 gm. This vapor carried away 644 Calories (1110×0.58).

$$\frac{\text{Heat dissipated by vaporization}}{\text{Total dissipation of heat}} = \frac{644}{2644} = 24.2\%.$$

The same methods were used to determine the percentage of heat lost by vaporization of water by four patients. The first three were youths aged, 15 years, 18 years and 18 years who were convalescent from minimal tuberculosis of the lungs but who were still confined to bed. None of them had fever and they felt well. They lost, respectively, 24.3%, 24.8% and 24.8%.

The last subject, a man 47 years of age, was in the hospital because of Meniere's disease. He had no attacks during the study. The data obtained in this case are especially valuable since he was being underfed and lost 1787 gm. in the 25 days of observation. The calculation was further complicated by a negative nitrogen balance of 1.36 gm. daily. Nevertheless the calculated per cent of heat lost by the vaporization of water was 24.7 even though the metabolic mixture was vastly different from the diet.

The average value for this second group was 24.6%. The response of these subjects all but one of whom remained in bed was very nearly the same as that of the first group who were leading active lives.

The uniformity of performance of these twelve persons indicates that man displays a strong tendency to rid himself by vaporization of water of one-fourth of the heat being produced within his body, provided he is comfortable in regard

to his environment. These studies also show that this tendency is maintained even when there are large differences in the total amount of heat being removed (2200 Calories to 3600 Calories).

However, since we have thus far been dealing with averages of performances that extended over weeks, these data give us no information about the extent of the day to day variation in the percentage of heat removed by vaporization of water. In order to obtain information about this question, we made use of the large respiration chamber described in the preceding paper (Newburgh et al., '37). It was merely necessary to add to its equipment a large balance capable of weighing a human being to 1 gm. The balance was so placed that its pointer could be observed through the window. In order to make a weighing, weights in excess of the weight of the subject were placed on the counterpoise before the chamber was closed. The subject was supplied with a pan of known weight which he held on his lap and to which he added weights until the observer signaled him that balance had been achieved. The subtraction of the weight of the pan and its contents from the weight of the counterpoise gave the weight of the subject.

For 3 or 4 days before entering the chamber the subject received a fixed diet which in some experiments contained much more energy than he required; at other times less; and often was close to the maintenance requirement. The carbohydrate also varied widely in different experiments. The diet eaten within the chamber followed this same plan. Our purpose was to learn what effect these shifts would have on the oxidation of carbohydrate.

The subject entered the chamber the night before an experiment was to be performed. He was supplied with liquid and solid food in appropriate tight containers, all of which had been previously weighed as a single item. At the end of the period, the containers were weighed again to obtain the weight of the ingesta. Urine and feces were voided into weighed containers with tight covers. We were thus able to obtain

an accurate statement of the insensible loss of weight. When all was ready, the chamber was closed and the ventilating pump started. The next morning while the subject was still in bed, the spirometers were washed with chamber air, samples of air from the chamber collected and the ventilation stopped long enough to read the meters. Then the ventilation was begun again, the spirometers opened and the time noted. Next the subject was notified to arise, urinate and be weighed. The time at the end of the weighing was noted. All of the urine up to this time was voided into a separate container and not included in the period. The subject now occupied himself as he pleased during the day. The next morning while the subject was still in bed, samples of air were withdrawn from the chamber. Then the spirometers were closed, the ventilation stopped and the time recorded, simultaneously. The meter readings were made. Immediately thereafter the subject arose, voided and was weighed. The time of the weighing was noted. Samples were withdrawn from the spirometers for analysis.

In addition to obtaining the insensible loss of weight we determined the production of carbon dioxide, the absorption of oxygen and the urinary excretion of nitrogen. This permitted us to calculate the weight of the water vapor by means of the equation:

$$I.W. = I.L. - (CO_2 - O_2) \, ;$$

and the heat lost by vaporization of water (I.W. $\times$ 0.58).

From the carbon dioxide, oxygen and urinary nitrogen we calculated the heat production by the standard procedure. Since we wanted to compare the heat removed by vaporization with the total dissipation of heat, it was necessary to subtract the heat required to bring the cold food to body temperature from the heat production before applying the formula

$$\frac{I.W. \times 0.58}{\text{Total Heat Elimination}}$$

This was accomplished by having the subject record the volume and the temperature of fluids whenever he drank them.

Next we compared the heat production determined by indirect calorimetry with the value derived from the insensible loss of weight. To make this latter calculation the composition of the metabolic mixture was ascertained. The urinary nitrogen was used as a measure of the protein. It was assumed that the carbohydrate oxidized equalled the carbohydrate of the diet. The fat of the metabolic mixture was calculated by means of an equation suggested by Lavietes ('35). Its derivation follows:

$$\text{I.L.} = \text{I.W.} + CO_2 - O_2$$

$$\text{I.W.} \times 0.58 \times \frac{100}{25} = \text{Calories}$$

$$0.58 \times \frac{100}{25} = 2.32$$

$$\text{I.W.} = \frac{\text{Calories}}{2.32}$$

Calories $= 4.1\ C + 26.5\ N + 9.3\ F$, where C is carbohydrate, N is urinary nitrogen and F is fat.

$$(CO_2 - O_2) = 0.41\ C + 0.84\ N - 0.08\ F\ [4]$$

$$\text{I.L.} = \frac{4.1\ C + 26.5\ N + 9.3\ F}{2.32} + 0.41\ C + 0.84\ N - 0.08\ F$$

$$\text{I.L.} = 1.77\ C + 11.42\ N + 4.009\ F + 0.41\ C + 0.84\ N - 0.08\ F$$

$$\text{I.L.} = 2.18\ C + 12.26\ N + 3.93\ F$$

$$F = \frac{\text{I.L.} - (2.18\ C + 12.26\ N)}{3.93}$$

The heat produced by the oxidation of protein, carbohydrate and fat was determined by multiplying them by their calorific values. Since I.L. had been lessened by a value corresponding to the heat transferred to the cold food, the appropriate number of calories was added to the heat of the metabolic mixture.

Finally we calculated how many grams of carbohydrate had been oxidized so that we could compare that value with the dietary carbohydrate.

We performed thirty-nine experiments on ten young men and women. Each subject was studied two or more times. One of them (O.M.) was in the third trimester of normal pregnancy. The remainder were presumably healthy persons leading active lives. The significant data are presented in tables 2 and 3. Four of the thirty-nine experiments were

[4] See page 209.

TABLE 2

Data with which heat production was calculated by indirect calorimetry and from insensible loss of weight

SUBJECT	DATE	O₂	CO₂	URINE N	NON-PROTEIN R.Q.	I.L.	CARBO-HYDRATE FED	HEAT TRANS-FERRED TO FOOD
		liters	*liters*	*gm.*		*gm.*	*gm.*	*calories*
B.DeV., M., age 25 years,	1–14–35	375.7	292.0	10.2	0.772	757	161	41
height 178 cm., weight 69 kg.	2–24–35	370.9	276.3	13.1	0.730	741	54	35
	3–24–35	440.1	327.1	16.5	0.725	817	96	38
	3–31–35	435.9	352.8	16.1	0.812	858	96	40
	4–30–35	379.6	288.4	11.8	0.750	667	91	45
	5– 1–35	399.3	303.3	11.2	0.751	687	91	41
	11–24–35	408.3	321.7	12.4	0.785	820	183	39
	11–28–35	381.3	323.0	12.5	0.857	807	183	40
	12– 8–35	404.6	329.0	11.8	0.816	893	85	42
	12–23–35	469.1	385.0	11.1	0.824	981	201	53
	2– 2–36	436.3	365.0	15.3	0.846	954	198	47
	2– 4–36	514.2	423.4	26.4	0.833	1097	50	55
	3– 6–36	554.7	423.3	13.9	0.756	989	53	30
	3–29–36	447.9	349.4	10.5	0.777	837	201	40
McQ., F., age 25 years,	2–28–35	334.4	259.7	14.0	0.769	756	52	43
height 163 cm., weight 60 kg.	3–21–35	372.2	271.5	15.1	0.707	675	78	56
Wm.M., M., age 30 years,	3–10–35	445.7	339.6	15.7	0.752	1292	100	60
height 177 cm., weight 70 kg.	4–22–35	455.4	332.9	11.9	0.718	926	95	63
	12–26–35	531.0	399.7	16.4	0.742	1115	53	52
	1– 2–36	495.6	388.9	11.7	0.782	1101	201	70
	1–12–36	526.2	422.0	13.0	0.802	1123	190	70
J.S., M., age 29 years,	10–10–35	418.2	311.9	12.6	0.733	952	59	32
height 168 cm., weight 64 kg.	11– 6–35	425.1	317.3	13.0	0.734	907	89	39
	11–12–35	396.8	325.2	12.5	0.824	870	183	42
	11–19–35	404.0	331.0	12.3	0.823	842	183	41
R.L.G., M., age 29 years,	11–21–35	442.8	360.4	11.7	0.816	1086	183	49
height 174 cm., weight 69 kg.	12– 5–35	466.6	373.9	13.0	0.801	1084	225	49
	12–12–35	443.6	333.2	14.3	0.739	1015	56	50
	12–19–36	476.7	358.5	15.9	0.740	1015	53	46
	1–30–36	430.8	365.7	12.0	0.858	1138	239	52
H.A., M., age 23 years,	10–24–35	406.7	293.1	12.8	0.702	907	59	25
height 180 cm., weight 62 kg.	3– 4–36	393.4	309.2	14.8	0.782	838	117	21
L.W.P., F., age 27 years,	2– 9–36	366.7	294.9	9.5	0.803	809	60	31
height 156 cm., weight 55 kg.	3– 1–36	352.0	272.0	11.0	0.766	728	60	27
M.B., F., age 29 years,	1–15–36	281.5	232.7	8.9	0.832	672	54	29
height 153 cm., weight 45 kg.	1–22–36	300.5	243.5	7.2	0.812	589	61	36
O.M., F., age 17 years,	1–19–36	327.1	297.7	10.6	0.936	722	230	60
height 156 cm., weight 53 kg.	1–24–36	345.9	288.2	10.7	0.840	778	205	60
D.S.R., F., age 29 years,	2–18–36	304.6	241.3	9.2	0.790	697	61	25
height 156 cm., weight 46 kg.	2–26–36	306.3	241.5	9.6	0.785	556	61	25

TABLE 3

Per cent of total heat lost by vaporization in the chamber and comparison of the calculation of heat production from insensible loss of weight with indirect calorimetry

1	2	3	4	5	6	7	8	9	10	11	12
	SUBJECT DATE	DIET Preparatory Calories	Carbohydrate	In chamber Calories	Carbohydrate	CARBOHYDRATE OXIDIZED	HEAT LOST BY VAPORIZATION PER CENT OF TOTAL	HEAT PRODUCTION Indirect calorimetry	From I.L.	DIFFERENCE BETWEEN 9 AND 10	REMARKS
		gm.	gm.	Calories	gm.	gm.		Calories	Calories	%	
	B.DeV.										
1	1-14-35	2006	161	2006	161	88	24.2	1772	1636	− 7.7	Cold 3 hours; relative humidity 71%
2	2-24	2000	54	2000	54	29	24.5	1729	1696	− 1.9	Relative humidity 71%
3	3-24	2450	98	2250	96	27	23.2	2059	1827	−11.3	
4	3-31	2456	98	1900	96	154	22.6	2065	1928	− 6.5	
5	4-30	2250	94	1602	91	57	21.5	1782			
6	5-1	1602	91	1602	91	63	21.5	1874			
7	11-28	2912	189	1845	183	112	23.7	1929	1755	− 9.0	
8	11-28	3005	396	1845	183	199	23.3	1828	1725	− 5.6	
9	12-8	3005	396	1850	85	154	25.4	1925	2035	+ 5.4	
10	12-23	1906	201	1906	201	203	23.7	2242	2134	− 4.7	Two quiet preliminary days; I.L.'s 933, 948, 981
11	2- 2-36	2374	198	2374	198	189	24.6	2079	2054	− 1.2	
12	2- 4	2374	198	3034	50	194(?)[1]	24.4	2430	2531	+ 4.2	
13	3- 6	2742	53	2742	53	100	21.4	2610			Cold during night
Average, omitting nos. 5, 6, 13							24.0			− 3.8	
	McQ.										
1	2-28-35	1793	54	1566	52	67	27.7	1567	1743	+11.2	
2	3-21	2009	81	1758	78	3	23.4	1725	1537	−10.9	Cold during night
Average							25.6			+ 0.2	
	Wm.M.										
1	3-10-35	2526	102	2065	100	68	36.2	2087	2124	+11.2	Sensible perspiration; relative humidity 60%
2	4-22	2627	101	1865	95	18	26.0	2124	2596	0.0	Relative humidity 60%
3	12-26	3260	60	2134	53	65	26.0	2486	2434	+ 4.2	
4	1- 2-36	3204	207	2213	201	137	26.7	2346	2493	+ 3.8	
5	1-12	3531	196	2386	190	185	25.0	2501		0.3	
Average, omitting no. 1							25.7			+ 1.9	

J.S.											
1	10–10–35	2251	94	1431	59	38	27.6	1935	2196	+13.5	Relative humidity 59%
2	11– 6	2987	200	1844	89	40	26.6	1987	2059	+ 3.1	
3	11–12	2952	190	1890	183	162	25.1	1889	1876	− 0.7	
4	11–19	2915	189	1884	183	165	23.7	1924	1808	− 6.0	
Average							25.8			+ 2.5	
R.L.G.											
1	11–21–35	2912	189	1884	183	175	28.5	2110	2387	+13.1	
2	12– 5	3340	303	2118	225	158	27.2	2215	2344	+ 5.8	
3	12–12	2969	60	2090	56	49	28.8	2054	2358	+14.8	
4	12–19	3259	60	2134	53	54	26.4	2229	2353	+ 5.6	
5	1–30–36	3210	243	2158	239	234	29.8	2070	2461	+18.9	
Average							28.1			+11.6	
H.A.											
1	10–24–35	2532	101	1422	59	0	28.3	1889	1844	− 0.4	Too warm
2	3– 4–36	3015	123	2022	117	99	25.1	1852		− 0.4	
Average, omitting no. 1							25.1			− 0.4	
L.W.P.											
1	2– 9–36	828	60	828	60	128	25.5	1743	1858	+ 6.6	
2	3– 1	828	60	828	60	73	24.8	1656	1658	+ 0.1	
Average							25.2			+ 3.3	
M.B.											
1	1–15–36	2004	64	942	54	123	27.3	1343	1539	+14.6	
2	1–22	2000	64	1400	61	117	22.7	1415	1348	− 4.7	
Average							25.0			+ 5.0	
O.M.											
1	1–19–36	2085	230	2085	230	260	23.4	1594	1486	− 6.0	
2	1–24	2085	230	1753	205	162	25.7	1654	1656	+ 0.1	
Average							24.6			− 3.0	
D.S.R.											
1	2–18–36	2004	64	1400	61	90	27.0	1441	1586	+10.3	
2	2–26	2004	64	1400	61	84	21.2	1447	1252	−13.4	
Average							24.1			− 1.6	

[1] This experiment was calculated in the standard manner, but it is probable that the carbohydrate oxidized is incorrect since the subject was grossly overfed and may have converted some carbohydrate to fat.

unsuccessful because the subjects were uncomfortably cold or warm. A fifth one is excluded because the relative humidity was so great that it presumably hindered the evaporation of water.

The remaining thirty-four experiments reveal a variation in the per cent of heat removed by vaporization of water from 21.2 to 29.8. This degree of inconstancy in the percentage of heat vaporized by persons residing in the chamber, raises the question whether the conditions imposed by it are in part responsible for the variation. Demonstrable evidence of an emotional disturbance associated with confinement in the chamber was recorded in the case of R.L.G. He had been one of the subjects of the earlier studies already described (Newburgh, Wiley and Lashmet, '31). Under those conditions when his activities were not hampered, he removed 25.2% of the heat by vaporization. But in the chamber, the results of the five periods ran from 26.4% to 29.8%. These high values were accompanied by a diuresis and a sharp loss of weight even though he was adequately fed. Since the response of this subject was in sharp contrast to that of all the others, we have excluded him when dealing with the averages cited below.

The variation exhibited by the nine other subjects was from 21.2% to 27.7%. The average value is 25.1%.

When the means of all the periods for each of the nine subjects are examined it is found that they all fall within the limits of 24.0% and 25.8%. The close agreement of the average performances of the nine subjects emphasizes the desirability of using the mean of several 24-hour periods whenever heat production is calculated from insensible loss of weight.

In that calculation it is assumed that the individual has lost 25% of the heat by vaporization. Hence an actual loss by vaporization of 24 or 26% would cause an error of ±4% in the final calculation.

The calculation of total heat from insensible loss of weight is also exposed to a second error since it is assumed that the carbohydrate oxidized is the same as the carbohydrate fed.

The significance of this potential error may be judged by means of the following considerations. When solving the equation,

$$\text{I.W.} = \text{I.L.} - (CO_2 - O_2)$$

the numerical value of $(CO_2 - O_2)$ is in part obtained by multiplying the dietary carbohydrate by 0.41. Accordingly I.W. is lessened by 0.41 per gram of carbohydrate assumed to have been oxidized. The effect upon the heat production calculated from I.L. is, therefore, $0.41 \times 0.58 \times \frac{100}{25} = 0.95$ calorie per gram difference between the carbohydrate ingested and oxidized. Our conception of the relationship between the ingestion and oxidation of carbohydrate is this. The individual whose liver glycogen is depleted will store large amounts of the ingested carbohydrate in the liver, even if the inflow is small. A person who possesses an unusually large store of liver glycogen may convert some of it to glucose as a source of energy even if the ingestion of carbohydrate is liberal. The actual amounts of glycogen lost or gained by the liver will be importantly influenced by the expenditure of energy and by the calories of the diet. An individual who expends approximately the same amount of energy daily and who takes a fixed diet will come into carbohydrate balance within a few days and remain so as long as the conditions persist. This last condition could only be established for the chamber experiments by restricting the subject's activity for some days before he entered the chamber and to feed a fixed diet throughout. We have only one experiment of this kind, that is no. 10 for the subject B. DeV. It will be seen that he did in fact oxidize the carbohydrate of the diet while in the chamber, as closely as it can be calculated.

In the remainder of the experiments, the activity of the subjects was in no way restricted during the preliminary days. Under these circumstances, none of the dietary plans that were tried could be relied upon to establish carbohydrate balance during the 24 hours in the chamber.

These experiments make it clear that the calculation of heat production from a single 24-hourly insensible loss of weight, cannot be depended upon to give a precise answer. Such a single determination does, nevertheless, yield a first approximation which we have found useful in the formulation of dietary plans. When, however, the average of the 24-hourly losses for each subject (omitting R.L.G.) is the basis of the calculation, the values obtained for the heat production by this method differed less than 5% from the figure determined by indirect calorimetry.

The data that we have reported thus far were obtained from normal subjects or patients whose regulation of heat was presumably normal. It will be interesting to learn whether edema, anaemia or heart failure causes a shift in the percentage of heat removed by vaporization. Clearly the method will give unsatisfactory results when the internal temperature is rising or falling. Several investigators have thought that rapid large shifts in the water content of the body are reflected in a change in the percentage of heat lost by vaporization. We have produced dehydration to the extent of 6% of the body water without causing a shift in the percentage of heat lost by vaporization (Newburgh and Johnston, '34).

We do not offer this method of measuring heat production as a substitute for either the calorimeter or the respiration chamber but rather as a means of extending the range of measurement. The calculation of heat from insensible loss may be satisfactorily carried out in individuals who continue to lead their customary lives provided they do not indulge in strenuous exercise or heavy manual labor. The method was successful in the case of the two graduate students in chemistry who produced approximately 3500 Calories daily. The second advantage of measuring heat in this way is that it permits one to obtain a continuous record for as many consecutive days as is desired. The simplicity of the equipment required is a third advantage.

Lavietes ('35), who has published a critique of the method, makes the following statement:

That Newburgh's experiments show remarkable agreement between actual and predicted metabolism over long periods of time can not be doubted. Under restricted conditions the insensible perspiration may yield valuable information concerning the energy metabolism which can be obtained otherwise in no way short of direct calorimetry. It seems extremely doubtful, however, ... that the method is applicable during conditions of changing hydration, or of value in the study of edema or diuresis.

Lavietes' distrust of the method when body fluids are shifting rapidly is largely based on the unsatisfactory results obtained by him. For example, he obtained his own 48-hourly insensible losses for 20 days while he pursued his ordinary activities. From the eighth through the thirteenth day he took 40 gm. of urea daily. The average of the losses during the three corresponding periods was 11% less than the average of the preceding and subsequent ones. Since Lavietes believes that the heat production was not diminished to a corresponding degree, he assumes that the method failed. Such a conclusion is extremely hazardous since he had no actual measurement of his heat production at any time. The following data indicate how greatly small movements of which one is not aware, influence the total heat production. In eleven 24-hourly periods, the heat production of a highly trained subject living in the respiration chamber, determined by indirect calorimetry, varied from 1729 Calories to 2242 Calories. The subject, questioned at the end of each period, was quite unable to predict the outcome.

SUMMARY

The results of this study are based upon observations made upon twenty different individuals. Most of them were young normal adults. The remainder were patients whose regulation of heat was presumably normal. Nine of them were studied in detail in the respiration chamber; four others while they remained in bed, and the remainder while they were pursuing active lives.

The chamber permitted us to measure the percentage of heat removed by vaporization during 24 hours. There are twenty-nine such periods (omitting R.L.G.) from nine subjects. The lowest per cent was 21, the highest 28. The average of the twenty-nine periods was 25%. In the two earlier series of experiments, the conditions permitted only a determination of the average per cent of heat removed by vaporization. Here the least was 23.8% and the greatest 25.2%. The average was 24.5%. The grand average for the whole series is 24.7%. Since biological variation is evident, we have adopted the nearest whole number which is 25%.

This narrow range is ample support for the belief that man possesses a mechanism designed to rid him by vaporization of water of a fixed percentage of the heat produced within his body. Conditions that cause him to feel uncomfortably warm or cool will disturb this relationship. The discomfort is the signal to adjust himself to the environment. He strives to do this by lessening or increasing the barrier between himself and the environment, by changing the rate of his activity or by making the surroundings more suitable.

This mechanism has been used as a basis for the calculation of the heat produced by normal human beings whose activity varied from continuous residence in bed to that of the busy laboratory worker. It is evident from the experiments in the chamber that the percentage of heat removed by vaporization of water during a single 24 hours was not sufficiently fixed to afford a reliable determination of the total heat. However, when the average of several periods was used, the maximal errors (omitting R.L.G.) attributable to variability in the per cent of heat lost by vaporization was only ±5%.

The second potential error in this calculation is due to the assumption that the carbohydrate oxidized is the same as the dietary carbohydrate. This error may be reduced to a negligible quantity by 1) feeding a fixed diet; 2) establishing a fixed plane of activity; 3) discarding the first few days of the study; 4) using the average of several consecutive days following the period of adjustment.

The calculation of heat production from the insensible loss of weight, the carbohydrate of the diet and the urinary nitrogen by the technique described above yielded values that differed less than 5% from those obtained by indirect calorimetry, when averages of several 24-hourly periods were used.

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IMPROVED GROWTH IN RATS ON IODINE DEFICIENT DIETS

ROE E. REMINGTON

Department of Nutrition, Medical College of the State of South Carolina, Charleston

In previously reported work of this laboratory on the results of iodine deficiency in the rat (Remington and Levine, '36), the low iodine diet employed has been as follows: wheat gluten 20, yellow corn meal[1] 76, calcium carbonate 3, sodium chloride 1, irradiated yeast 0.2. Young rats maintained for 35 days on this diet show the following changes in the thyroid: 1) enlargement, 2) hyperplasia, 3) edema, 4) almost complete loss of colloid, and 5) marked loss of iodine. Since these changes are fully prevented by the addition of iodine (1 to 2 γ per day) either as iodides or certain iodine-bearing foods (Remington, Coulson and Levine, '36) they are considered as specific consequences of lack of this element.

This diet falls far short of satisfying the ideal for the study of deficiencies, i.e., full adequacy in all known essentials except the one being investigated. Glaringly evident are the unbalance between Ca and P, and the deficiency in vitamins of the $G(B_2)$ group. Probable deficiencies also exist in inorganic elements, and in the quality of the protein. It does not support normal growth. Rats 4 weeks of age, averaging 60 gm. in weight, when placed on the diet for 5 weeks, with or without added iodide, grow only to 110 to 130 gm., as compared with 180 to 200 gm. (average for both sexes) for rats on our colony ration. Attempts to rear rats on this diet to full size and sexual maturity have not been successful.

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[1] Whole yellow corn meal as sold for chick feeding.

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Obviously, if one desires to answer such questions as to whether or not normal growth can be obtained in iodine deficiency, or what is the effect of deficiency continued into adult life on the animals themselves, on the composition and histology of the thyroid, or on reproduction, the first step must be an attempt to improve the diet without notably decreasing its goiter-producing power. Many other problems affecting the utilization of iodine in the rat can be attacked in a quantitative way, if such a diet is possible. Difficulties have been the universal presence of traces of iodine in natural foods or concentrates prepared from them, and the presence of significant traces in many chemically pure salts. It should be borne in mind that a degree of contamination which will augment the iodine intake of the rat 0.1 to 0.2 γ per day can produce a detectable difference in the size of the thyroid and its iodine content. For instance, if a given supplement is to be added to the diet at a level of 2%, it should not contain more than 0.00005% (500 p.p.b.) iodine, since this amount would add approximately 0.1 γ per day to that unavoidably present in the basal diet. It is, on the other hand, quite probable that if a given supplement improves growth, and the economy of food utilization for growth, the daily intake of iodine necessary to maintain a given degree of thyroid abnormality may be altered, in which case our ideas as to iodine requirement (Levine, Remington and von Kolnitz, '33) will require revision, since these were developed on rats which made subnormal growth.

<center>EXPERIMENTAL</center>

The first step in improving the diet was to counteract its rickets-producing property by reducing the proportion of $CaCO_3$ from 3 to 1%, it having been shown that this change did not lessen the degree of thyroid enlargement. This change permitted the omission of irradiated yeast. The new basal diet (no. 327) is as follows: wheat gluten 20, yellow corn meal 78, $CaCO_3$ 1, NaCl 1. Experiments were then made in supplementing the diet in three ways, viz., 1) improvement in protein quality by substituting purified (vitamin-free) casein

for part or all of the wheat gluten; 2) improvement of vitamin and mineral supply by addition of small percentages (0.5 to 4%) of dried pig liver, or 3) by the addition of similar small percentages of dried brewers yeast.

The handling of the animals was essentially the same as that used in earlier experiments. Young rats, approximately 28 days of age and weighing around 60 gm., were placed on screens and maintained on the diets with distilled water for 35 days, after which they were killed by chloroform and the thyroids removed. Records were kept of the gain in weight and of the food consumed. Ten rats, either five of each sex or four of one and six of the other, were placed on each diet.

The glands on removal were placed in special nickel boats, and weighed in a stoppered weighing bottle. All of the glands from a given group were placed in the same boat, which was then dried to constant weight at 100°, for calculation of dry matter. Iodine was determined colorimetrically on the entire contents of the boat by the method of the writer (Remington, McClendon, von Kolnitz and Culp, '30).

Effect of casein

Results obtained when vitamin-free casein was substituted for part or all of the wheat gluten are shown in table 1. Five per cent of casein, substituted for wheat gluten, produces a significant gain in rate of growth, which still is only about half that of normal rats. Further increases in proportion of casein do not bring about any greater gains. When the thyroid is considered, however, it appears that with increasing proportions of casein there is a progressive decrease in thyroid weight, and an increase in dry matter and iodine content, the data conforming well to our curves (Remington and Levine, '36). Either the casein carries a significant amount of iodine, or its presence in the diet makes possible a higher degree of storage in the thyroid of the small amount of the element available in the diet. The effects observed can be attributed to increased iodine supply if the casein contains 200 to 300 parts per billion, a point which will be discussed later.

Effect of liver

Fresh pig liver was obtained from a distributor, sliced, partially dried on a steam table, ground through a meat chopper, the drying completed in an oven at 80°, then reground and stored in tightly sealed jars. The first series of experiments was run parallel to the casein experiments, with

TABLE 1

Effect of casein

DIET	327	328	329	330	COLONY
Wheat gluten	20	15	10	0	
Casein	0	5	10	20	Commercial
Corn meal	78	78	78	78	dog
CaCO₃	1	1	1	1	biscuit
NaCl	1	1	1	1	
Number of rats	10	10	10	10	10
Sex	4 M–6 F	4 M–6 F	5 M–5 F	3 M–7 F	5 M–5 F
Gain in weight (gm.)	51±2	68±4	74±4	67±4	120
Thyroid weight (mg.)	27.4 (19.1–37.7)	24.9 (18.2–36.1)	23.1 (18.2–27.9)	18.3 (15.1–21.1)	15.8 (11.9–20.2)
Thyroid weight per 100 gm. body weight (mg.)	23.9±1.0 (18.7–35.2)	18.9±0.6 (13.7–21.9)	17.1±0.5 (13.6–21.6)	14.1±0.4 (11.4–16.5)	8.8±0.4 (6.3–11.7)
Dry matter (%)	21.0	23.6	25.9	27.4	32.0
Iodine, dry basis (%)	0.0137	0.0297	0.0451	0.0595	0.2000
Total iodine (γ)	0.79	1.75	2.70	2.99	10.1
Degree of enlargement	2.4	1.9	1.7	1.4	

the same control group. Dried liver was added to the diet at levels of 1.5 and 3%. Both groups showed greater growth than the casein-fed rats, but unlike the latter, these had considerably larger thyroids, of lower iodine content, than the controls. Since work in this direction appeared to show promise, a second series was run (table 2), the liver being fed this time at levels of 0.5, 1.0, 2.0 and 4.0% of the diet, replacing in each case the same amount of gluten. In addition, a group

of rats was fed 2% of dried lean beef (chuck). In this series, maximum growth was obtained at the 2% liver level, as was also maximum degree of thyroid enlargement (3.7 times normal weight). Beef muscle also stimulated growth, but to less than

TABLE 2

Effect of liver. Series II

DIET	327	340	341	342	343	344	COLONY
Wheat gluten	20	19.5	19	18	16	18	
Dried liver	0	0.5	1	2	4	0	
Corn meal	78	78	78	78	78	78	Com-
CaCO$_3$	1	1	1	1	1	1	mercial
NaCl	1	1	1	1	1	1	dog
Dried lean beef	0	0	0	0	0	2	biscuit
Number of rats	10	10	10	10	10	10	
Sex	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	
Gain in weight(gm.)	66 ± 3	81±2	92±3	104±4	105±5	85±4	125
Thyroid weight(mg.)	30.4	34.0	42.3	52.1	46.1	37.3	15.8
Thyroid weight per 100 gm. body weight (mg.)	23.9±1.0	23.9±1.0	27.8±1.4	32.3±1.7	28.2±1.1	25.6±0.8	8.8±0.4
Dry matter (%)	22.4	21.8	21.3	20.9	21.1	22.4	32.0
Iodine, dry basis (%)	0.0064	0.0049	0.0032	0.0051	0.0034	0.0055	0.2000
Total iodine (γ)	0.44	0.36	0 .29	0.55	0.33	0.46	10.1
Degree of en-largement	2.4	2.4	2.8	3.7	3.2	2.6	......

half the degree of liver, and the thyroids were not significantly larger than the controls (2.6 times normal against 2.4). In all cases the iodine content of the thyroid is so low that no importance can be attached to such differences as exist.[2]

[2] The total amount of iodine contained in the dried thyroids of ten animals was 3 to 5 γ, which cannot be estimated by present methods with any higher degree of accuracy than about ± 20%.

Effects of yeast

In the third series (table 3) dried brewers yeast, furnished through the courtesy of Dr. Harold Levine of the Pabst Brewing Company, was added to diet 327 in the proportions of 0.5, 1.0, 2.0 and 4.0% replacing an equal amount of gluten. Growth is improved by the yeast, most marked effect being observed at the 2% level, but no significant changes in thyroid weight, dry matter, or iodine content are observed. Neither was growth as good as with equal additions of liver.

TABLE 3

Effect of brewers yeast

DIET	327	345	346	347	348
Wheat gluten	20	19.5	19	18	16
Dried yeast	0	0.5	1	2	4
Corn meal	78	78	78	78	78
CaCO$_3$	1	1	1	1	1
NaCl	1	1	1	1	1
Number of rats	10	10	10	10	10
Sex	5 M–5 F	5 M–5 F	5 M–5 F	5 M–5 F	5 M–5 F
Gain in weight (gm.)	54±2	64±4	67±3	80±4	84±4
Thyroid weight (mg.)	22.1	26.1	25.6	28.1	27.9
Thyroid weight per 100 gm. body weight (mg.)	19.2±0.6	18.5±0.8	19.9±1.0	19.7±0.8	19.1±0.5
Dry matter (%)	24.1	25.2	24.8	24.9	24.8
Iodine, dry basis (%)	0.0084	0.0074	0.0083	0.0067	0.0086
Total iodine (γ)	0.45	0.49	0.53	0.47	0.60
Degree of enlargement	1.9	1.9	2.0	2.0	1.9

DISCUSSION

The four supplements fed; casein, liver, beef muscle and yeast, all improved the growth of the animals, but in different amounts. Rats receiving 5% of casein in place of the same amount of wheat gluten gained 17 gm. more than the controls, but this gain was exceeded by those receiving 1% of dried liver, or 2% of beef muscle or yeast. Since casein is a widely used and satisfactory protein in experimental diets, it does not seem likely that the greater improvement in growth resulting from much smaller additions of the other supplements

can be due to protein quality alone. It is possible that the vitamin content, particularly with regard to A and G, of the corn meal, is insufficient for rapid growth, and that this partial deficiency is compensated by the vitamins present in liver or yeast. Deficiency in iron is also a possibility, although rats on the basal diet do not become anemic within the experimental period employed. Even though the present investigation does not reveal specific deficiencies in the basal diet (327) it has achieved the desired end of producing normal, or nearly normal, growth on diets markedly deficient in iodine. An investigation of the effects of augmenting the vitamin content of diet 327 with carotene or flavin would contribute to the specificity of our knowledge as to how the liver acts, and might make possible a further simplification of the problem.

When it comes to the effect on the thyroid gland, i.e., degree of goiter as indicated by the criteria previously proposed: fresh weight, iodine content, and dry matter content of the gland, the response to different supplements is quite different. Casein added to the diet in progressively increasing amounts brought about a diminution in the severity of goiter in almost exact relation to the amount fed. Previous experience with adding iodides or iodine-bearing foods in quantities insufficient for full protection against goiter, enables us to calculate that if the effect is due to iodine in the casein, it should contain between 200 to 300 parts per billion. Two analyses of casein from the same source made several years ago revealed twelve and eighteen parts, respectively, an insignificant amount. It seemed possible therefore that unless casein itself is able to protect the thyroid against the results of iodine lack, either the earlier analysis must have been in error, or the present lot of casein differs in iodine content from previous ones. Accordingly, the particular lot of casein employed was analyzed, the chemist not being informed as to the purpose of the analysis or probable values.[3] Four determinations gave values of 250, 254, 259 and 267 parts per billion, amply con-

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[3] Iodine analyses on dietary materials were made by Mr. Cecil L. Smith, on thyroid glands by Mr. E. J. Coulson.

firming the belief that the reduction in degree of goiter obtained with casein is due to iodine contamination, and rendering it unnecessary to postulate any specific protective action due to casein.

The marked increase in severity of goiter when liver is added to the diet was not expected, and is rather disturbing when considered alongside the earlier work of this laboratory in which it appeared that quantitative relationships exist between iodine intake and degree of hyperplasia of the thyroid. The dried pig liver contained about 150 p.p.b. of iodine, enough to contribute 0.04 γ per day per rat when fed at the 2% level. This additional intake of iodine should have had a slight protective action, probably too small to measure. Instead of such an effect, however, the degree of enlargement of the glands of rats receiving 2% liver is 3.7 times normal, compared with 2.4 for those receiving no liver.

It was suggested earlier in the paper that augmented growth might conceivably increase the iodine requirement of the rat, and hence bring about a more severe thyroid condition on the same iodine intake. Fortunately, the yeast series bears on this question. Rats receiving 2% of yeast made the same gain as those receiving 1% of liver, about 26 gm. more than the controls in both cases. The yeast contained less than 20 p.p.b. of iodine, hence its contribution of that element was negligible. Nevertheless yeast did what we had expected the liver to do, increased growth without affecting the degree of thyroid enlargement. If, then, rate of growth can be increased by 50% by the addition of 2% of yeast, which neither increases nor decreases appreciably the iodine content of the ration, and this improved growth is not accompanied by an increased severity of the thyroid condition, we cannot with surety attribute such increased severity to the effect of growth when obtained by means of liver.

To explain the effect of liver on the thyroid, two possibilities are presented: 1) the presence in the liver of some substance, the metabolism of which causes a drain on the thyroid mechanism, or 2) the presence of a specific thyroid-stimulating

hormone. In Marine's early observations on goiter in young brook trout fed largely on chopped liver, he wrote ('14): "It is also probable that the liver contains some substances in excess, in attempting to utilize which the animal exhausts other elements necessary for nutrition which are not present in the liver in sufficient amounts." This view is reflected

TABLE 4

Economy of food utilization for growth

DIET NO.	SUPPLEMENT	TOTAL GAIN	TOTAL FOOD	FOOD FOR GRAM GAIN
		Series I		
327	Basal	51	348	6.8
328	5% casein	68	335	4.9
329	10% casein	74	383	5.2
330	20% casein	67	382	5.7
331	1.5% liver	81	398	4.9
332	3.0% liver	87	417	4.8
		Series II		
327	Basal	66	385	5.9
340	0.5% liver	81	411	5.1
341	1.0% liver	92	437	4.8
342	2.0% liver	104	458	4.4
343	4.0% liver	105	475	4.5
344	2.0% lean beef	85	406	4.8
		Series III		
327	Basal	54	317	5.9
342	2.0% liver	99	411	4.2
345	0.5% yeast	64	332	5.2
346	1.0% yeast	67	348	5.2
347	2.0% yeast	80	368	4.6
348	4.0% yeast	84	346	4.1
Colony		120	482	4.0

in the first suggested explanation. Marine ('22) also states that "liver, particularly pig liver, was the most potent of a great variety of meats in causing thyroid hyperplasia in dogs and cats . . . .," but since his protocols have not been published, one cannot be sure that he has ruled out differences due to other deficiencies, such as iodine. Marine also believed that increased protein in the diet increases the tendency to goiter, but in the present work we have not appreciably varied

the percentage of protein, hence the experiments do not bear on this point.

All the supplements fed, in addition to improving growth, also increased the economy of food utilization for growth, as shown in table 4. If we disregard the small amount of fat contained in the liver and yeast, the caloric value of all diets was the same. The most efficient utilization of food was found in rats fed 2% liver or 4% yeast: 4.2 and 4.1 gm. of food, respectively, for each gram of gain. On the basal diet alone it required 5.9 gm., while a typical group on the colony diet (dog biscuit) consumed only 4 gm. of food for each gram of gain.

From the foregoing it appears that diet 342, containing 2% of dried pig liver, should enable us to reach the desired end of prolonging our experimental period through a normal life span of the animals. Rats have been raised to maturity on this diet, with a progressively greater degree of thyroid hyperplasia (up to 5 months), have produced normal numbers of living young and reared them without loss to weaning age, despite the fact that their thyroids were markedly hyperplastic, contained practically no colloid after 8 weeks, and only 3 to 5% as much iodine as those of rats on colony ration.

SUMMARY

The subnormal growth of young rats fed the goiter-producing diet of Levine and Remington is improved when a part of the wheat gluten is replaced by 1) purified casein, 2) dried pig liver, or 3) dried brewers yeast. Equal gains were produced with 10% casein, 1% liver or 2% yeast. The addition of 2% liver produced most satisfactory growth.

Purified casein carries sufficient iodine to render it unsuitable for use in goiter-producing diets. Yeast was without effect on degree of goiter but liver aggravated the condition.

A suitable diet for goiter studies on rats is wheat gluten 18, dried pig liver 2, yellow corn meal 78, calcium carbonate 1, sodium chloride 1. On this diet rats reach maturity and produce normal numbers of living young despite almost complete absence of iodine and colloid from the thyroid gland.

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A STUDY OF THE EFFECT OF VITAMIN B AND IODINE ON THE WEIGHT, IODINE CONTENT AND STRUCTURE OF THE THYROID GLAND OF THE RAT

MARY DORIS CARPENTER AND GEORGE R. SHARPLESS

Henry Ford Hospital, Detroit, Michigan

Cowgill ('32) and Hendricks ('33) have suggested that the vitamin $B(B_1)$ requirement of an animal is proportional to its metabolism. The thyroid gland, through its iodine containing hormone (thyroxin), has been shown to provide a means both for maintaining a higher rate of metabolism than would otherwise be obtained and for varying the rate of metabolism to meet changing physiologic needs.

It has been shown that the function of the thyroid can be limited by a deficiency of iodine. McClendon ('27) and Orr ('31) have reviewed the subject of goiter and its relation to iodine supply. Studies of the effect on the thyroid of cabbage feeding (Webster and Chesney, '30), of methyl cyanide (Marine, Rosen and Cipra, '33), of calcium (Hellwig, '34) and of chlorides (Hibbard, '33) have indicated that a number of factors may increase the needs of the body for iodine or for the iodine containing hormone and thereby may play a part in the development of simple goiter.

A number of workers have associated thyroid function with the vitamin B complex and lately with the $B(B_1)$ fraction. McCarrison ('14) noticed hyperplasia of the thyroid of pigeons fed a diet of polished rice. The suggestion has been made (Aalsmeer, '32) that beri-beri is associated with dysfunction of the thyroid. Stepp and György ('27) state that

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vitamin B deficiency causes loss of colloid and hyperplasia of the cells lining the follicles. Experiments on both pigeons and dogs indicate that there is a relation between vitamin B requirement and metabolism (Cowgill and Palmieri, '33). A similar reaction has been pointed out by Hendricks ('33). McCance ('33) has stated that vitamin B along with iodine, is one of the essentials in the cure of certain goiters. According to Sandberg and Holly ('33) vitamin B causes the involution of goiter produced in rabbits by cabbage feeding, but not to the degree caused by iodine. Fischer ('33) concluded from microscopic findings that the antineuritic vitamin of unpolished rice produced the same histological effect on the thyroid as iodized salt.

Thus it is seen that a relation between vitamin B and the thyroid has been indicated but has not been studied critically. It was our purpose to make such a study, namely: the effect of vitamin $B(B_1)$ on the thyroid of the rat as shown by weight, iodine content, and structure of the gland when the rat was fed well-controlled purified diets at different levels of iodine intake.

EXPERIMENTAL

The rats used in this study were bred on an adequate stock ration of the following composition: whole wheat flour, 30.0 parts; yellow corn meal, 30.0 parts; whole milk powder, 30.0 parts; linseed meal, 5.0 parts; alfalfa meal, 2.7 parts; wheat germ 1.0 part; calcium carbonate, 0.6 part; sodium chloride, 0.6 part. (A short time previously, iodized salt and 3 parts cod liver oil had been used, but these were discontinued because the young were getting such a large store of iodine.) Twenty-five animals were placed on each diet. Rats of both sexes, 26 days old and weighing between 60 and 70 gm., were used. Animals which were fed concentrates were kept in individual cages; otherwise two or three were caged together. Distilled water was given to all animals and a record of food consumption was kept for each cage.

With certain modifications iodine was determined by the Leitch and Henderson ('26) method. The food materials were ashed by the von Kolnitz and Remington ('33) method while

the thyroids were burned in a combustion tube 1 inch in diameter. It was found that good checks could be obtained with the combustion tube with sugar and potassium iodide and with samples of desiccated thyroid if the oxygen was passed through slowly. A tube of small diameter or a rapid flow of oxygen caused the smoke to be swept through, and check results could not be obtained.

Iodine determinations were made on the diet materials and on one lobe of the thyroid of each rat. Differences as small as 0.10 γ of iodine could be detected and in samples containing 0.5 γ or more the error was less than 10%.

At the end of the experimental feeding period the rats were killed with chloroform and the thyroid glands were weighed in a moist chamber. The glands were obtained by removing the trachea and thyroid intact and then pulling the trachea and attached muscles free, while the gland was held between the operating board and a scalpel which was forced between it and the trachea. If proper care is exercised the gland is obtained complete and free from muscle. One lobe was placed in formalin for sectioning, the other was dried and saved for iodine determination. The iodine content of the whole gland was calculated from the content of this one lobe.

The weight of the thyroid was recorded in three ways: 1) actual weight; 2) weight per 100 gm. of body weight; 3) per cent of Donaldson's ('24) normal values.

Three different age groups were studied as follows: 1) 41 days on experimental diet; 2) 82 days on experimental diet; 3) 123 days on experimental diet. Forty-one days were chosen as the initial period because that was the time necessary for our rats to show marked vitamin B deficiency. The other two periods were chosen as multiples of the initial 41-day period. The animals receiving the deficient diets were given some yeast supplement in order to sustain life for the longer periods.

Diets. The composition of the experimental diets and the groups into which they were divided are shown in table 1.

Results. The results are summarized in table 2, a study of which reveals some interesting findings that can best be brought out when discussed under the following headings:

TABLE 1

Showing composition of the diets and the groups into which they were divided

DIET NO.	GROUP NO.	VITAMIN FREE CASEIN[1]	AUTOCLAVED BREWER'S YEAST	BREWER'S YEAST (NOT AUTOCLAVED)	SUCROSE[2]	BUTTER-FAT	SALTS NO. 185[3]	VITAMIN G (B$_2$) CONCENTRATE[4]	VITAMIN B (B$_1$) CONCENTRATE[5]	KI	NATURE OF DIET
		per cent	per cent	per cent	per cent	per cent	per cent			per cent	
32	...	12.0	0	0	79.0	5.0	4.0	0	0	0	Def. in B complex and I$_2$
33	...	12.0	0	0	79.0	5.0	4.0	0	0	0.005	Def. in B complex 0.0038% I$_2$
38	I	12.0	5.0	0	74.0	5.0	4.0	0	0	0	Def. in B$_1$ and I$_2$
32G	I	12.0	0	0	79.0	5.0	4.0	+	0	0	Def. in B$_1$ and I$_2$
39	II	12.0	5.0	0	74.0	5.0	4.0	+	0	0.005	Def. in B$_2$, 0.0038% I$_2$
33G	II	12.0	0	0	79.0	5.0	4.0	+	0	0.005	Def. in B$_2$, 0.0038% I$_2$
39A	III	12.0	5.0	0	74.0	5.0	4.0	0	0	0.025	Def. in B$_2$, 0.019 I$_2$
40	IV	12.0	0	5.0	74.0	5.0	4.0	0	0	0.005	Complete diet, 0.0038% I$_2$
39B	IV	12.0	5.0	0	74.0	5.0	4.0	0	+	0.005	Complete diet, 0.0038% I$_2$
40A	V	12.0	0	5.0	74.0	5.0	4.0	0	0	0.025	Complete diet, 0.019%I$_2$
41	VI	12.0	0	5.0	74.0	5.0	4.0	0	0	0	Complete diet, no. I added
38B	VI	12.0	5.0	0	74.0	5.0	4.0	+	+	0	Complete diet, no. I added
32BG	VI	12.0	0	0	79.0	5.0	4.0	+	+	0	Complete diet, no. I added
33BG	IV	12.0	0	0	79.0	5.0	4.0	+	+	0.005	Complete diet, 0.0038% I$_2$

To all of the above diets 15 drops (2800 U.S.P. units) of viosterol were added per kilogram of food mixture.

[1] Casein purchased from Casein Manufacturing Company.

[2] Cane sugar used in preference to other carbohydrates because of its more uniform action in causing symptoms of vitamin B deficiency.

[3] McCollum's.

[4] Vitamin G(B$_2$) concentrate prepared from yeast according to the directions of Dr. N. B. Guerrant, as follows: One kilogram of yeast was extracted with water and alcohol added to the extract to make 50% by volume. The formed precipitate was discarded. Additional alcohol was added until a concentration of 80% by volume was obtained. The precipitate formed between these two concentrations was removed, dried, autoclaved to destroy any vitamin B present, dried again, and kept in a desiccator. An amount equivalent to 1 gm. of the original yeast was fed to each animal daily.

[5] The vitamin B concentrate was prepared from wheat germ by extracting with acidified (0.1% glacial acetic acid) 95% alcohol and evaporating the extract under reduced pressure. It was fed daily at a level equivalent to 1 gm. of the original wheat germ per rat. All fatty and waxy materials soluble in ether were removed from the wheat germ before leaching with alcohol.

TABLE 2

Showing average iodine intake, body weight, thyroid weight, iodine content and concentration of thyroid

DIET NO.	GROUP NO.	IODINE CONTENT OF DIET	AVERAGE TOTAL INTAKE PER ANIMAL	INITIAL AND FINAL BODY WEIGHT	THYROID WEIGHT				IODINE CONTENT OF THYROID			
					Actual	Per 100 gm. body weight	Per cent of Donaldson's values	Actual average	Fresh weight basis	Dry weight basis	Per 100 gm. body weight
		γ/kilo.	γ	gm.	mg.	mg.		γ	per cent	per cent	γ
41 days on experimental diet												
38	I	<20	<2.1	70– 56	5.9	11.1	52	4.4	0.074	0.24	7.8
32G	I	<20	<3.8	59– 46	4.0	8.7	38	5.8	0.16	0.34	12.6
39	II	38220	6268	74– 57	4.9	8.6	40	4.8	0.099	0.29	8.4
33G	II	38220	6738	58– 45	4.0	8.9	38	5.68	0.14	0.35	12.6
39A	III	191000	43853	63– 66	5.9	8.9	44	10.13	0.17	0.53	15.3
40	IV	38220	8775	63–102	4.5	4.4	23	24.9	0.55	1.51	24.4
39B	IV	38220	12849	62–104	7.3	6.8	42	8.97	0.13	0.36	8.62
40A	V	191000	59515	61– 95	7.4	7.6	41	26.05	0.36	0.86	27.4
41	VI	<20	<6	70–138	5.2	3.8	21	6.64	0.13	0.36	4.8
38B	VI	<20	<6.5	63–107	6.8	6.5	37	8.98	0.14	0.35	8.4
82 days on experimental diet												
38	I	<20	<5.7	68– 68	6.4	9.9	49	4.6	0.069	0.17	6.8
39	II	38220	11580	75– 80	6.0	7.5	38	7.07	0.117	0.36	8.8
39A	III	191000	73535	63– 76	5.8	7.6	38	10.14	0.17	0.47	13.3
40	IV	38220	29773	75–163	7.1	4.4	26	23.27	0.33	0.78	14.3
39B	IV	38220	25683	62–123	7.4	6.0	33	8.20	0.11	0.30	6.7
40A	V	191000	137711	59–144	7.5	5.2	30	22.11	0.29	0.68	15.3
41	VI	<20	<12	66–165	8.8	5.3	32	8.82	0.10	0.24	5.3
38B	VI	<20	<13	60–118	7.5	6.3	35	9.20	0.12	0.34	7.8
Stock				266	13.8	6.1	34	7.50	0.053	0.17	2.8
123 days on experimental diet												
38	I	<20	<8	69– 90	6.9	7.6	39	5.70	0.083	0.22	6.3
39	II	38220	20218	72– 94	5.7	6.0	31	10.54	0.19	0.50	11.2
39A	III	191000	70479	61– 53	5.1	9.6	44	9.74	0.19	0.66	18.4
40	IV	38220	48883	68–193	8.6	4.6	27	32.6	0.38	0.83	16.9
40A	V	191000	213786	64–167	9.8	5.5	32	31.12	0.34	0.74	18.6
41	VI	<20	<20	67–169	9.1	5.8	35	17.7	0.18	0.57	10.5
Stock				319	18.0	5.8	38	9.3	0.053	0.19	2.9

a. Appearance of the thyroid gland. Rats in group I consistently showed glands that were dark red in color with engorgement of the associated blood vessels. The rats in all the other groups had glands that were either light red or very pale in color. The variations in appearance could not be correlated with any histological difference or with any difference in iodine content.

TABLE 3

Showing average thyroid weight and iodine content of rats on paired feeding experiments

DIET NO.	GROUP NO.	IODINE CONTENT OF DIET	AVERAGE TOTAL INTAKE PER ANIMAL	INITIAL AND FINAL BODY WEIGHT	THYROID WEIGHT			IODINE CONTENT			
					Actual	Per 100 gm. body weight	Per cent of Donaldson's values	Actual average	Fresh weight basis	Dry weight basis	Per 100 gm. body weight
Average of three sets of litter mates											
		$\gamma/kilo$	γ	*gm.*	*mg.*	*mg.*	%	γ	%	%	γ
38	I	<20	2.5	67–55	3.9	7.8	35.1	4.7	0.12	0.28	8.5
39	II	38220	4777	63–45	3.7	8.6	35.9	4.3	0.12	0.34	9.6
40	IV	38220	4777	64–45	4.4	8.5	42.7	4.7	0.11	0.26	10.4
41	VI	<20	2.5	64–45	3.8	8.6	37.5	4.4	0.12	0.27	10.0
Average of three sets of litter mates											
32G	I	<20	1.7	64–39	4.9	12.4	53.4	3.9	0.09	0.30	10.0
33G	II	38220	3248	62–39	4.6	11.6	50.1	4.1	0.09	0.30	10.5
33BG	IV	38220	3248	60–42	4.7	9.9	42.9	4.5	0.10	0.31	10.7
32BG	VI	<20	1.7	61–50	4.1	9.8	42.5	5.0	0.11	0.34	10.0

b. Comparison of fresh thyroid weights. Forty-one-day period. When the groups are compared on the basis of the average actual weight of the gland it is found that there is not a statistically significant difference between any of the groups. When comparison is made on the basis of weight of gland per 100 gm. of body weight, the glands of rats in group I (low vitamin B and low iodine) are significantly larger than those of rats in groups IV and VI which were fed vitamin B. The question of whether this was an actual enlargement due to vitamin B deficiency or a relative enlargement due to starvation was answered by paired feeding studies shown in table 3.

They show that starvation rather than a deficiency of vitamin B causes the relative increase in weight of the gland and indicate that in this study comparisons should be made of the actual weight of the glands.

Eighty-two- and 123-day periods. In each of these two periods, there was no significant change from the 41-day period. The actual weight of the glands of rats fed the vitaman B deficient diets remained nearly constant, while the weight of the glands of rats fed the vitamin B containing diets increased slightly with the increase in body weight.

The value for each group, including the stock animal group, is less than 50% of the value given by Donaldson ('24) for normal rats of the same weight.

c. Iodine content and concentration of the glands. The glands of rats in group I had an iodine content of approximately 5.0 γ and a concentration of approximately 0.2%, dry basis, in each of the three periods. This was the lowest concentration found in any of the groups and is more than twice the 0.075% necessary (Remington and Levine, '36) to induce enlargement of the gland. However, it is within the range where hyperplasia should occur in some cases. The average iodine content of the thyroid of rats when weaned was 4.5 γ so that in group I the iodine contents were just maintained. The glands of rats in all of the other groups had higher concentrations of iodine as shown in table 2.

d. Vitamin B and thyroid iodine concentration. With a comparable iodine intake the thyroids of rats fed the diet containing dried yeast (diet 40) had a higher concentration of iodine than those of rats fed the diet containing autoclaved yeast (diet 39A) or the diet containing autoclaved yeast and vitamin B extract (diet 39B). The values in the above order for the 41-day period were 1.5% ± 0.3%, 0.53% ± 0.05%, and 0.36% ± 0.06% iodine calculated on the dry basis. In the 52-day period the values were 0.78% ± 0.12%, 0.47% ± 0.05%, and 0.30% ± 0.01% iodine. The values for the 123-day period were 0.83% ± 0.23% iodine for diet 40 and 0.67% ± 0.04% iodine for diet 39A. Values were not obtained for diet 39B in the 123-day period.

In the 123-day period the difference in iodine concentration of the glands of rats fed diet 40 and those of rats fed diet 39A is not significant. The higher concentration in the glands of rats fed diet 39A may have been caused by the dried yeast that was fed in order to keep them alive for the 123 days. It is shown in table 2 that the growth was practically the same for rats on diet 40 and diet 39B, which indicates that there was not a deficiency of vitamin B in diet 39B and therefore the lower thyroid iodine concentration is not a result of vitamin B deficiency. The results suggest that some factor other than vitamin B is present in dried yeast which affects the thyroid iodine concentration and that this factor is lost when the yeast is autoclaved.

e. Microscopic findings. Stock. The acini of most of the glands contained medium staining colloid and were lined with cuboidal epithelium (fig. 1). Seven glands out of thirty-three showed slight hyperplasia.

Group I. The glands of rats in this group were similar to those of the stock animals (fig. 2). Three out of twenty showed a slight hyperplasia.

Group II. Seventeen of the nineteen glands examined had dilated acini with low cuboidal epithelial lining and dense pink staining colloid filling the lumina. The appearance was that of moderate colloid goiter (fig. 3). The other two glands were slightly hyperplastic.

Group III. Eleven glands out of twelve appeared normal, the twelfth being slightly hyperplastic.

Fig. 1 Normal gland of rat from stock colony. The acini are of average size, lined by columnar epithelium and the lumina contain lightly staining colloid. (Age, 110 days.)

Fig. 2 Group I, diet 38 (low iodine, low vitamin B). Numerous small acini lined or filled with cuboidal cells. The larger acini have lumina filled with lightly staining colloid material. (Age, 108 days.)

Fig. 3 Group II, diet 39 (low vitamin B, 0.0038% iodine). Shows large dilated acini filled with darkly staining colloid, also smaller acini lined with cuboidal epithelium. The condition resembles colloid goiter. (Age, 108 days.)

Fig. 4 Group VI, diet 41 (basal diet with vitamin B but no I_2). Small acini filled with columnar cells. The few lumina present are filled with lightly staining colloid. (Age, 108 days.)
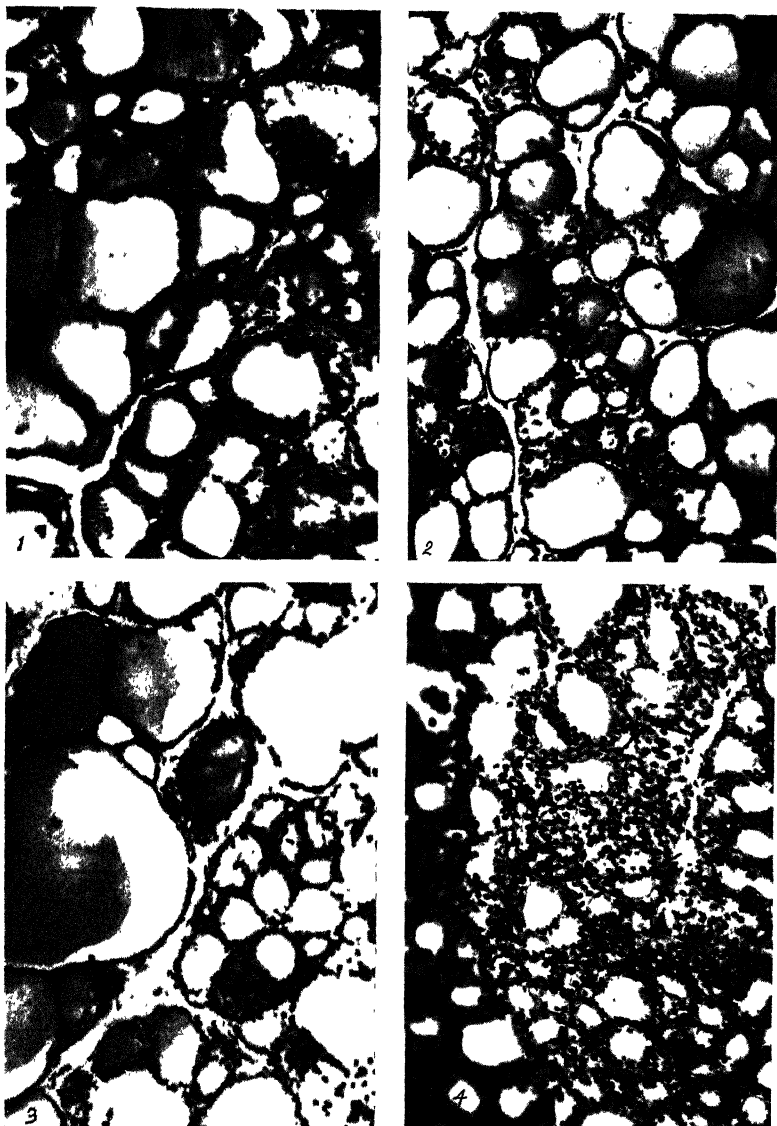
Figures 1 to 4

Group IV (diet 40). Twenty-one glands out of twenty-seven had acini filled with dense pink staining colloid and six were slightly hyperplastic.

Diet 39B. Twelve glands out of fourteen were normal; two were slightly hyperplastic.

Group V. The glands in this group were very similar to those in group IV. Five out of eighteen were slightly hyperplastic.

Group VI (diet 39B). Seven glands out of twelve were normal. Five showed varying degrees of hyperplastia, in no case extensive.

Diet 41. Eleven glands out of twenty-four were hyperplastic (fig. 4), with acini varying in size and shape; in many instances the smaller ones were filled with cells. The larger acini were lined with cuboidal or columnar epithelium and contained lightly staining colloid.

f. Condition simulating colloid goiter. Seventeen out of nineteen rats examined in group II, fed the vitamin B deficient diet containing 0.0038% iodine, had thyroid glands which microscopically simulated colloid goiter. Group II was the only group in which this condition occurred. Rats in group I, fed the same diet with no iodine added, had glands similar to those of the stock animals. Rats in group II, fed the same diet with 0.019% iodine, also had normal glands. Rats in group IV, which received vitamin B in addition to the diet used in group II, had thyroids which also resembled those of the stock animals.

The vitamin B was supplied as dried yeast in diet 40 and as an extract of wheat germ in diet 39B (table 1). The additional iodine supplied by these sources of the vitamin was negligible. As near as could be determined by the method used, the iodine content of dried yeast was the same as that of autoclaved yeast. Iodine was determined in the combined diet and extract and gave the same value as the diet alone. Six rats in group IV with food intake limited to the amount eaten by littermates in group II also had normal glands.

Hellwig ('34) has produced colloid goiter consistently in rats by feeding 2 γ potassium iodide daily with a diet that normally produced a parenchymatous type of goiter. With 20 γ of potassium iodide daily, a normal sized gland, microscopically resembling resting colloid goiter seen in human subjects, was obtained. The effect of a higher iodine intake was not determined.

In this experiment goiter was not produced when iodine was not added to the diet. In the light of Hellwig's ('34) experiments, the production of a colloid goiter-like condition in such a large per cent of the rats in group II is therefore surprising and difficult to explain. If there is a relation between the vitamin B requirements and metabolism as has been shown by Cowgill and Palmieri ('33), Hendricks ('33), and others, it is possible that in vitamin B deficiency the demands made on the thyroid are so small that a tendency to goiter under the conditions of this experiment would not show up. Another possible explanation would be that in vitamin B deficiency an intake of iodine greatly in excess of the physiological requirement will cause a condition which microscopically resembles colloid goiter.

Study of the hypophysis

Since a relation is known to exist between the hypophysis and the thyroid, the hypophyses of all animals studied were weighed in the same manner as has been described for the thyroid. In each period, the glands of rats fed diet 41 were the largest, but the individual variation was so great that the enlargement was not significant.

SUMMARY

1. Vitamin B deficiency alone did not affect the size, structure, or iodine content of the thyroid gland of the rat.

2. Rats fed a diet deficient in vitamin B and containing 0.0038% iodine developed a thyroid gland that microscopically simulated colloid goiter. This condition did not develop when the diet contained 0.019% iodine or vitamin B.

3. The results suggest that some factor present in yeast which is lost when the yeast is autoclaved and which is not supplied by a vitamin B containing extract causes an increase in the iodine content and concentration of the thyroid.

4. Differences in the weight of the hypophysis of rats on the different diets were not statistically significant.

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THE COMPOSITION OF MILK FROM STOCK RATS AND AN APPARATUS FOR MILKING SMALL LABORATORY ANIMALS [1]

WARREN M. COX, JR. AND ARTHUR J. MUELLER

Research Laboratory, Mead Johnson & Company, Evansville, Indiana

TWO TEXT FIGURES AND ONE PLATE (ONE FIGURE)

In spite of the fact that the rat is widely employed in laboratory investigations, very little is known concerning the composition of its milk. Hatai ('17) obtained milk from the stomachs of suckling young, but apparently in quantities insufficient for complete analysis. More recently, Mayer ('35) has reported the composition of milk obtained by the same method. In spite of these studies, it must be noted that the mixing of milk with salivary and gastric juices, even for the shortest possible time, may result in considerable change in composition.

We have been successful in milking rats directly. More than 600 cc. of milk from stock rats has thus been made available, and in the present communication we report the complete analysis of milk from stock rats.

METHODS AND RESULTS

Because of the limited amount of milk that may be obtained from an individual rat, pooled samples were used for all analyses. We first determined the changes in composition at various stages of lactation; arbitrarily, at the eighth, thirteenth, eighteenth and twenty-fourth days of lactation. The

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[1] Presented at the meeting of the American Society of Biological Chemists, Washington, D. C., March, 1936.

results are reported in table 1. Total solids were determined by heating a weighed sample at the temperature of boiling water until almost dry, and then placing in a vacuum oven at 75°C. for 4 hours; total ash, by heating an evaporated, weighed sample at 550°C. in an electric muffle; total nitrogen, by the standard Kjeldahl with selenium added to speed digestion (Lauro, '31); fat, by extraction with alcohol and ether in a Mojonnier and Troy ('25) apparatus, and lactose, by iodometric titration of the oxidized Cu_2O (Methods of Association of Official Agricultural Chemists, '30 (a)) using the

TABLE 1

Composition of rat milk at various stages of lactation

	DAY OF LACTATION				
	8th	13th	18th	24th	Average
Specific gravity $\frac{25}{25}$	1.049	1.044	1.046	1.047	1.047
Solids (as determined), %	31.02	32.87	27.13	35.86	31.7
Total nitrogen × 6.38	11.81	11.71	11.70	(11.85)	11.8
Fat	14.03	16.05	12.14	16.93	14.8
Carbohydrate	2.40	3.05	3.40	2.46	2.8
Ash	1.70	1.48	(1.50)	(1.36)	1.5
Solids (sum)	30.0	32.3	28.7	32.6	30.9

Parentheses indicate that more than one pooled sample was used in obtaining an average value.

filtrate from the differential precipitation of casein and lactalbumin (Methods of Association of Official Agricultural Chemists, '30 (b)) after fat extraction.[2]

In spite of the fact that, in the early stages of lactation, adequate milk for analysis was obtained only with difficulty (raising the question of evaporation), no great differences in composition were noted. This may be more readily understood if it is remembered that 1 day in the life of the rat is equivalent to 1 month of human life (Donaldson, '24). Eight days after parturition, lactation in the rat, therefore, is rather completely established. At the period of greatest milk production (eighteenth day after parturition), total solids are

[2] A known amount of lactose was added to a sample of rat milk. After performing the indicated separations, the lactose was quantitatively recovered.

slightly less than at other periods—reflecting a slight drop in fat content.

In view of the high nitrogen content of rat milk, a study of the nitrogen distribution was considered desirable. A condensed summary of such studies is given in table 2, and it is to be noted that the percentage distribution of the nitrogenous constituents does not differ greatly from that recorded for

TABLE 2

Protein distribution in pooled samples of rat milk. (*The prime figures indicate the number of determinations*)

SAMPLE NO.	VOLUME OF SAMPLE	TOTAL N × 6.38 [a]	TOTAL PRECIPITATED PROTEIN [b]	CASEIN (METHODS, A.O.A.C., '30) [b]	ALBUMIN	GLOBULIN (MOIR, '31)	N.P.N. [d]	ALBUMIN + GLOBULIN [c]	CASEIN + GLOBULIN (MOIR, '31)
	cc.	%	%	%	%	%	%	%	%
1	29	9.57^6	89.2^3	79.8^3	6.11^2		13.6^4	13.3^1	
2	38	10.68^9	89.0^6	78.7^3	7.49^1		11.8^6	12.4^2	
3	45	10.66^{12}	89.3^3	78.2^6	9.85^2		11.2^7	12.7^3	
4	49	10.22^{12}	89.4^9	76.9^4	7.60^6	4.8^4	11.2^6	13.4^2	82.8^3
Average	..	10.28^e	89.2	78.2	7.76	4.8	11.9	12.9	82.8
Cow (Davies, '32)	..	3.17	94.1	76.1	12.6	5.4	5.9	18.0	...

[a] Average of N determinations on whole milk and of the sum of the separate nitrogenous constituents in each complete set of partitions, × 6.38.

[b] Average of total protein precipitated by trichloracetic acid, and the sum of the individually partitioned protein precipitates in each complete set of observations.

[c] Precipitated with trichloracetic acid after separation of casein.

[d] Expressed in terms of percentage nitrogen × 6.38.

[e] Analyses made in late spring and early summer. The effect of season may account for the difference between this figure and those reported in tables 1 and 3.

cow's milk. Casein and albumin were determined by the Methods of the Association of Official Agricultural Chemists ('30 (b)), save that in the analysis of the fourth sample, these methods were supplemented by the procedures of Moir ('31). Non-protein nitrogen was determined on the filtrates only after the addition of trichloracetic acid.

In table 2 it will be noted that the percentage of total nitrogen as casein, and as globulin, in cow's and rat's milk, is approximately equal; but that the percentage of total nitrogen as albumin is less in the latter in spite of a larger absolute amount (table 3). Conversely, non-protein nitrogen ($\times$ 6.38) is greater when expressed as percentage in milk—or as percentage total nitrogen, in rat's milk than in cow's milk.

The composition of rat milk, as compared with that of cow and human milk, is given in table 3. The percentages saturated and unsaturated fatty acids were determined by the lead salt ether method (Jamieson, '32 (a)), iodine number by the Wijs method (Jamieson, '32 (b)), and saponification number in the usual way (Jamieson, '32 (c)). The Association of Official Agricultural Chemists procedures were followed for the Reichert-Meissl and Polenske values (Methods, Association of Official Agricultural Chemists, '30 (c)). Calcium in the ash was determined by the micro method of Tisdall and Kramer (Peters and Van Slyke, '32 (a)); magnesium by the 8-hydroxy quinoline method of Greenberg and Mackey ('32), sodium as the uranyl zinc sodium acetate (Butler and Tuthill's modification of Barber and Kolthoff's method (Peters and Van Slyke, '32 (b)), and potassium by difference, after determination of total alkali sulphates (Peters and Van Slyke, '32 (c)). Total phosphorus was determined colorimetrically after precipitation with strychnine molybdate, according to Tisdall (Peters and Van Slyke, '32 (d)); chloride by determination of silver nitrate required for complete precipitation (Peters and Van Slyke, '32 (e)). Iron was determined by the method of Winter ('31), and copper by the method of Supplee and Bellis ('22).

The tenfold concentration of iron and copper in rat's milk, as compared with cow's milk and human milk, is worthy of note. Similarly, calcium, phosphorus, and magnesium are present in three times the concentration that they are in cow's milk; whereas, sodium, potassium and chlorine are present in amounts strictly comparable with that present in cow's milk.

TABLE 3

Comparison of the composition of rat, cow and human milk

	RAT MILK		COW MILK		HUMAN MILK	
Specific gravity	1.047		1.031[1]		1.031[1]	
Protein, %	11.77		3.42[9]		1.25[1]	
Lactalbumin Calc'd,		0.91		0.56[9]		0.75[1]
Casein table		9.20		2.86[9]		0.50[1]
N. P. Nitrogen × 638 3		1.33		0.20[11]		....
Carbohydrate, %	2.83		4.75[1]		7.50	
Fat, %	14.79		3.50[1]		3.50[1]	
% saturated acids		46.5[3]		67.4[4]		43.5[7]
% unsaturated acids		48.4		27.4[4]		55.1[7]
Av. mol. weight saturated acids		248.2		....		258.0[7]
Av. mol. weight unsaturated acids		267.8		....		.....
Saponification number		231.0		230.5[3]		205.1[6]
Iodine number		41.0		32.0[3]		56.2[6]
Reichert-Meissl number		4.9		28.0[3]		2.5[6]
Polenske number		11.7		2.3[3]		0.1[6]
Refractive index		1.461[20°]		1.455[40°3]		1.457[40°7]
Ash	1.50		0.75[1]		0.20[1]	
Potassium		0.170		0.154[1]		0.048[1]
Sodium		0.076		0.061[1]		0.011[1]
Chloride		0.117		0.116[1]		0.036[1]
Calcium		0.349		0.122[1]		0.034[1]
Phosphorus		0.272		0.090[1]		0.015[1]
Magnesium		0.031		0.013[1]		0.005[1]
Iron		0.0007[5]		0.00007[1,5]		0.0002[1,5]
Copper		0.0007[5]		0.00006[5]		0.00005[10]
Ca/P ratio		1.28		1.35		2.26
Total solids (sum)	30.89		12.42		12.45	
Total solids (determined)	31.7		....		....	
pH	6.6		6.7[1]		7.0[1]	
Buffer capacity[8]	109.0		42.0		13.0	
Lysozyme content	Rat milk contains more than cow milk, less than human milk[5]					
% calories, as protein	24.6		21.7		7.5	
% calories, as fat	69.5		48.8		47.4	
% calories, as carbohydrate	5.9		29.5		45.1	

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[7] Roller, P. E., J. Pediatrics, vol. 4, p. 238, 1934.

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[10] Hess, A. F., G. C. Supplee and B. Bellis, J. Biol. Chem., vol. 57, p. 725, 1923.

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We question whether these differences are suggestive in relation to the physiological requirements for rapid growth and the fact that rats are cartilagenous at birth.

In view of the fact that our stock diet contained 65% of the fat as butter fat, the difference in the Reichert-Meissl and Polenske numbers of butter fat as compared with the fat of rat's milk should not pass unnoticed. In partial explanation, at least two factors influence slightly the composition of milk fat—the season of the year, and the food fed. Changes in composition are noted primarily in the degree of unsaturation and the percentage volatile acids (Davies, '36 (a)). Ingested volatile fatty acids, below lauric, are presumably metabolized by the animal and are not manifested by significantly increased Reichert-Meissl or Polenske values (Hilditch and Sleightholme, '30). To a large extent, therefore, the percentage volatile acids in a milk may be regarded as characteristic of the species.

In planning experimental diets, it is of some theoretical interest to know the distribution of calories ordinarily ingested by the suckling. It will be noted (table 3) that the diet of the young rat contains some 69% of the total calories as fat, and but 5.9% as carbohydrate. In the usual experimental diet, a large percentage of the energy requirement is supplied by carbohydrate.

Buffer curves of rat, cow, human, and guinea pig milk before and after dilution were determined by means of the micro-quinhydrone electrode (Cullen, '29) using a type K, Leeds and Northrup potentiometer. The resulting curves are given in figure 1. The high buffer quality of rat's milk is but another way of expressing the degree of concentration of milk protein and milk salts.

SUMMARY

1. More than 600 cc. of milk from stock rats, obtained by means of a micro-milking device, have been analyzed. Milk solids are approximately three times as concentrated as in cow's milk. Compared to human and cow's milk, rat milk is low in carbohydrate, and high in protein and fat.

2. The average composition of rat milk obtained at various stages of lactation is: total solids, 31.7%; protein, 11.8%; fat, 14.8%; carbohydrate, 2.8%; and ash, 1.5%.

3. Studies on the distribution of nitrogen in the total protein, on the constants of the extracted butter fat, and on the composition of the ash of rat's milk are reported.
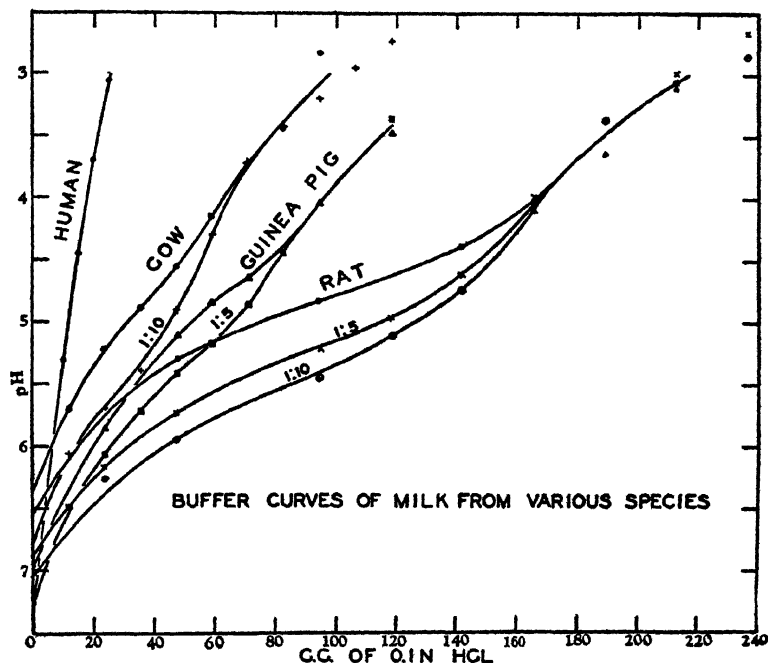
Fig. 1 Buffer curves of rat, guinea pig, cow, and human milk. The potentiometric determinations were made on diluted and undiluted samples. The ratios (1: 10, etc.) indicate the degree dilution; the acid was equally diluted in each case.

4. The buffer values of rat and guinea pig milk have been determined, and are compared with similar values for cow's milk and human milk.

ADDENDUM

With the thought that other investigators may wish to study the effect of drugs, hormones, surgical procedures or dietary

changes on the composition of rat milk, we give, in the following brief paragraphs, a description of our technic.

In milking, at least three factors must be considered: 1) the support of the small, collapsible teat, 2) the negative pressure applied to the end of the teat (which, if excessive, may cause congestion), and 3) the number of alternating pulsations (suction applied and released) per minute.

Teat cup. A small rubber tube, tapered, seemed to be the most suitable teat cup, especially as the degree of taper would determine the amount of support for the teat. The large end of a no. 10 French, soft rubber catheter has considerable taper, and in addition, the thickness of the wall decreases from about 1 mm. to almost zero. The inside diameter of the tube increases from 1 mm. to about 6 mm. By applying various negative pressures, and cutting off segments from the more collapsible end of the catheter, we eventually determined the degree of suction (7 inches Hg) that would collapse the tube around the teat, and permit milk to flow from the end of the teat. The number of pulsations per minute was not overly important and could vary from 25 to 40.

The application of the cup to the rat's teat, and the alternation of negative pressure from a water pump required the presence of two operators. An automatic device, operable by one individual, was eminently desirable.

Automatic milking device. To take the place of the assistant, an automatic cut-off for the suction line from the water pump was essential. After several unsuccessful arrangements a magnetic switch (220 volts A.C.) (after removal of all accessories so as to leave only the electro-magnet and the movable arm), proved satisfactory. A copper wire, attached to the movable arm, was passed over a piece of flexible rubber tubing (connected as a by-pass to the water pump and teat cup) held firmly between two segments of stationary glass tubing. When the switch is energized, the arm moves downward, crimps the rubber tubing and prevents inlet of air. Release of the arm permits the rubber tube to regain its original shape and admit air. The switch is enclosed in a wooden box to reduce noise.

Alternating pulsations were obtained by connecting a mercury U-tube (one arm sealed) with a suitable relay (20,000 ohms), and the magnetic switch. In order to allow adequate lag in pulsation (a drop from 7 inches negative pressure to zero pressure, before the switch was re-energized) two adjustable electrodes were placed at different heights in one arm of the U-tube. By sticking a small nail through a cork,
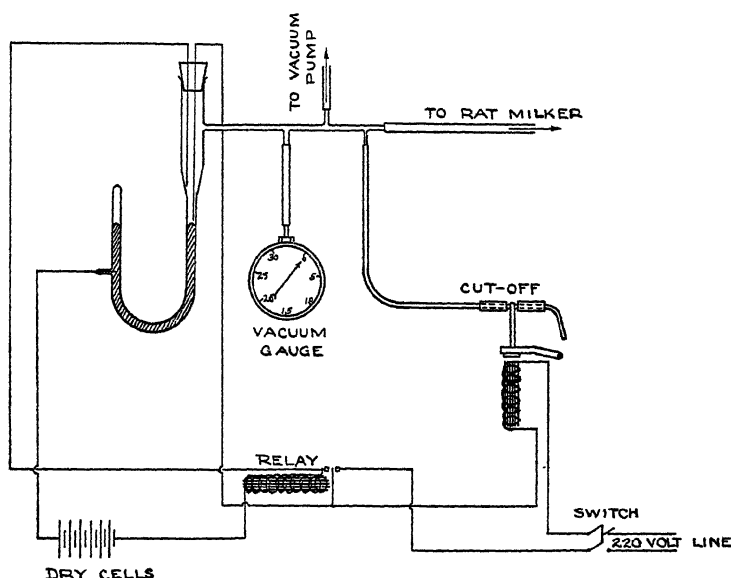
Fig. 2 Diagram of electrical connections for rat milking apparatus

the null-point of the relay became a contact point.[3] The electrical connections are shown in figure 2. When the mercury made contact with the higher electrode, the magnetic switch was de-energized, and it was not re-energized until the mercury had fallen below the lower electrode.

Suction was obtained from an ordinary water pump, but a vacuum line serves even better as a source of negative pressure.

[3] We are indebted to Mr. Lee Stone of Mead Johnson & Company for assistance in the electrical design.

A picture of the machine is given in plate 1. The apparatus may be successfully used in milking rats and guinea pigs. We have not used it on other laboratory animals.

Preparation of rats. Obviously, milk must be in the glands before any can be obtained. The amount of milk available varies with many factors, and we list in the table below those conditions which are preferred in order to obtain the largest possible amount of milk from our laboratory stock rats (inbred Wisconsin strain):

Weight, 300 gm. or better
Reproductive cycle, at least the third
Number of young, 8 to 10
Day of milking, sixteenth to twenty-second
Absent from young, 12 to 16 hours

Milking procedure. To obtain milk from rats the operator holds the rat (grasped from the back) in his left hand in an almost vertical position, the thumb and index finger holding the two front legs apart. With the right hand (placed between the hind legs in order that the rat may support herself) the teat cup is applied to any one of the six centrally located teats. Those most available are the second and third thoracic, and the abdominal pair. The number of teats may vary from ten to thirteen (Myers, '16). We have observed several females with thirteen teats.

With the thumb and index finger of the right hand the operator gently strokes the breasts, in order to work the milk toward the teat. Slight pressure is exerted with each development of negative pressure by the machine. The perfection of this procedure is one of the principal essentials for successful milking.

Amount of milk obtained. The mother rat is removed from her young on the evening prior to milking. The next morning her breasts are usually filled with milk. We have not been successful in obtaining milk, night and morning, every day. One milking on two alternate mornings at the height of lactation is about all that can be expected, if one is not satisfied with small volumes. From about 10% of stock rats no milk at all was obtained.

The volume of milk varies with the stage of lactation, as indicated in the following table. This parallels the increasing amount of milk obtainable from the stomachs of nursing young (Mayer, '35).

Volume of milk obtainable at different stages of lactation

Day of lactation	Average volume per rat cc.	Maximum volume from a single rat cc.
7th– 9th	0.5	3.0
12th–14th	0.8	5.0
16th–18th	1.6	8.0
24th	0.7	5.0

By excluding rats with no milk in their breasts the average volume obtained at the sixteenth to eighteenth days of lactation can be increased to about 2.1 cc.

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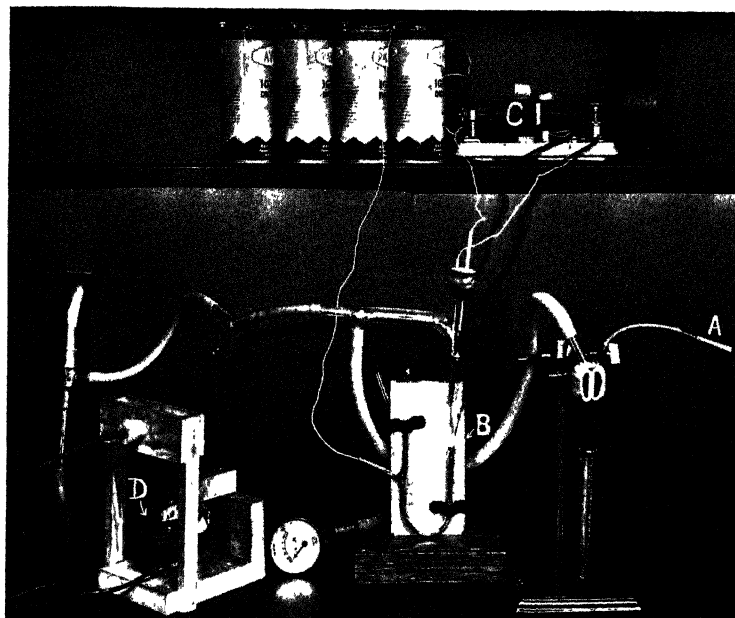
Photograph of rat milking machine. A, teat cup; B, mercury U-tube with two electrodes at different heights in one arm; C, 20,000 ohm relay; D, magnetic switch.

A CONSIDERATION OF THE NUTRITIVE STATE IN THE METABOLISM OF WOMEN DURING PREGNANCY [1]

FRANCES COPE HUMMEL, HELEN A. HUNSCHER, MARY F. BATES, PRISCILLA BONNER AND ICIE G. MACY

The Research Laboratory of the Children's Fund of Michigan

and

JOSEPH A. JOHNSTON

Department of Pediatrics, Henry Ford Hospital, Detroit

THREE FIGURES

Significant individual differences have been pointed out in the metabolic processes of healthy women (Macy, Hunscher, Nims and McCosh, '30; Hunscher, Donelson, Erickson and Macy, '34) and children (Hunscher, Cope, Noll, Macy, Cooley, Penberthy and Armstrong, '32; Hunscher, Cope, Noll and Macy, '33; Macy, Hunscher, Hummel, Bates and Poole, '36) as shown by the rate of retention of the acid-base mineral elements and nitrogen when the subjects were observed under similar dietary and environmental conditions. In view of these findings it becomes increasingly difficult to compare the metabolic responses of several individuals (Hunscher, Hummel, Erickson and Macy, '35) under the demands of preg-

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nancy without knowing something of the underlying factors governing them such as nutritive state and other physiological conditions.

Although Rubner so long ago showed that the nutritive state of the individual markedly influenced the rate of storage in the body, little attention has been given to it in past interpretations of metabolic balance data in determining dietary requirements of man and animal. Of the same diet more was retained when the nitrogen content of the dog was low than when it was high. In interpreting Rubner's findings, Lusk ('21) states that

According to these laws adult cells which have been depleted of their protein may gradually improve their nutritive condition until they reach an optimum, at which point they lose their power to attach additional protein.

In the case of calcium, phosphorus and iron there is a provision in some of the tissues for an inert deposit or bodily reserve (Bauer, Aub and Albright, '29) in contrast to nitrogen which has become an integral part of soft tissues of the body without extensive deposit. Fairbanks and Mitchell ('36) have recently shown in experiments with rats that

Differences in the degree of saturation of the skeletal tissues with respect to calcium, brought about by previous subsistence upon diets differing in their contents of this element produce inequalities in the retention of calcium under uniform conditions of calcium nutrition such that low saturation is associated with subsequent high retention of calcium. It follows that the rate of calcium retention by growing animals under conditions of adequate nutrition measures the requirement of calcium only when the calcium stores have been saturated by appropriate pre-feeding. Otherwise the observed calcium retentions will be greater than the day to day requirements of calcium.

Realizing the necessity for long time observations and appreciating the importance of previous nutritional state in metabolic balance studies, Clark ('26) pioneered the field in a most admirable manner. The success of his endeavors culmi-

nated in the demonstration of the effect of unfilled or depleted body reserves of five men who under strict metabolic regime stored during 28 continuous weeks nitrogen and the acid-base mineral elements in amounts characteristic of their bodies' former nutritive deprivations. With the calcium intake of approximately 0.90 gm. per day the per cent of the intake retained for the men were in the following ascending order: 3.1, 6.5, 17.2 and 30.6—findings which were commensurate with those predicted by Clark from his knowledge of the dietary habits of the men during previous years. Likewise, other recognized differences in human metabolic response decisively due to the nutritive state have been shown in under-nourished children by Stearns ('31), Wang, Kaucher and Frank ('28) and Wang, Kern and Kaucher ('29) as well as by Daniels, Hutton, Knott, Wright and Forman ('35).

The present report records the metabolic balances of an 18-year-old primapara who was observed uninterruptedly during the final 65 days of gestation. Her medical history showed an unsatisfactory nutritional background for the pre-ceding 6 years. For the purpose of determining if the nutri-tive state of the maternal organism is one of the causative factors for the differences observed among individuals under the physiologic influences imposed by pregnancy (Hunscher, Hummel, Erickson and Macy, '35; Hummel, Sternberger, Hunscher and Macy, '36) the data obtained have been com-pared with similar ones collected continuously to term on two healthy women during the same interval of reproduction. It is hoped that these data will contribute to the interpretation of the metabolic balance method for determining dietary re-quirements for pregnancy.

<center>EXPERIMENTAL PROCEDURE</center>

Subject. An 18-year-old girl of American descent who had been under the care of the staff in the Department of Pedia-trics of the Henry Ford Hospital, Detroit, Michigan, became available for metabolic observations during the last 65 days

of her first pregnancy.[2] She was maintained under highly controlled conditions of study in a private room in the hospital under the constant supervision of specific doctors, nurses and dietitians.[3] Being a ward of a child caring institution all of her life her food consumption and condition of work were not always optimal for a growing adolescent, and she was known to be slightly underweight reaching a pre-gravid body weight of approximately 48 kg. and a height of 158 cm.

During the study the subject appeared happy and serene and seemed to have a feeling of security. She was intelligent and alert in following directions concerning the consumption of food and fluids, as well as the collection of the urine and separation of it from the feces. There was genuine interest and desire on the part of the subject in making all observations successful.

Dietary. A series of diets was planned for 10 consecutive days so that the daily food intake was fairly constant in composition yet by substituting the usual common fruits, vegetables, cereals and meat in food equivalents not only interesting

but varied and appetizing menus were possible. The set of diets was rotated every 10 days to the complete satisfaction of the subject in spite of the regulated food intake. In addition, 1500 gm. of milk containing 125 U.S.P. units of vitamin D per quart and 20 gm. of cheese, the diet not only contained an abundance of all known nutritive principles but furnished approximately 2500 calories per day. A period of 2 weeks was given for adjustment to the diet and to the metabolic routine.

Procedure. The general metabolic procedure and methods of analyses as previously reported by Macy et al. ('30), Hunscher et al. ('35), and Hummel et al. ('36) have been followed. A special dietitian had charge of preparing and weighing of food served to the subject and of samples for analyses.

<center>RESULTS AND DISCUSSION</center>

Table 1 gives a summary of the metabolic data secured during the continuous study of thirteen successive 5-day balances made during the last 65 days of pregnancy. It shows the average daily ingestion, excretion in both the urine and feces, and the resultant maternal gain or loss of calcium, magnesium, sodium, potassium, phosphorus, sulfur, chlorine and nitrogen.

Individual trends in the daily storage of nitrogen and calcium are shown in figure 1 for the primapara (I.M.B.) and the quadripara (L.R.). With a mean intake of 261 mg. of nitrogen per kilogram of body weight the former retained 24.5 mg. while in contrast, the latter with a lower intake stored 38.5 mg. per day. In terms of total nitrogen exchange, the mean daily intake of I.M.B. and L.R. were 14.05 gm. (13.51 to 14.82 gm.) and 19.03 gm. (18.34 to 21.10 gm.) with a mean net daily storage of 1.32 gm. (0.32 to 2.22 gm.) and 3.02 gm. (1.64 to 5.44 gm.), respectively. It is of interest to compare these findings with the other similar studies on women found in the literature, namely, the classic case of Hoffström ('10) and that of Coons and Coons ('35), the latter being carried

TABLE 1

Nitrogen and acid-base mineral elements retained per day by a primipara (I.M.B.) who was studied uninterruptedly during the last 65 days of pregnancy

DAYS ANTE-PARTUM	BODY WEIGHT	NITROGEN				PHOSPHORUS				CHLORINE				SULFUR			
		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
65–69	52.0	14.82	12.46	1.18	1.18	2.09	1.28	0.64	0.17	4.59	4.64	0.05	−0.10	1.16	0.84	0.14	0.32
60–64	52.1	14.28	11.72	1.09	1.47	1.77	1.20	0.49	0.09	3.85	4.59	0.04	−0.78	1.16	0.69	0.14	0.34
54–59	52.3	13.67	11.92	0.98	0.77									0.94	0.67	0.09	0.17
50–53	52.6	14.02	10.96	1.10	1.96	1.90	1.28	0.50	0.12	4.25	3.86	0.05	0.34	1.04	0.63	0.10	0.31
45–49	52.8	13.96	11.80	0.95	1.21	1.83	1.14	0.40	0.29	4.26	4.71	0.02	−0.47	1.03	0.65	0.11	0.28
40–44	53.0	13.99	11.75	1.04	1.20	1.87	1.17	0.46	0.24	4.33	4.06	0.03	0.24	1.02	0.62	0.11	0.28
35–39	53.5	13.95	11.62	0.95	1.38	1.99	1.19	0.42	0.38	4.18	3.95	0.02	0.21	1.06	0.65	0.10	0.30
30–34	54.1	13.86	12.53	1.01	0.32	1.97	1.15	0.53	0.29	4.10	4.60	0.02	−0.52	1.09	0.66	0.14	0.29
25–29	54.4	14.13	11.21	0.70	2.22	1.95	1.04	0.39	0.52	4.15	3.99	0.04	0.12	1.06	0.63	0.11	0.32
20–24	54.9	13.51	10.95	1.02	1.54	2.01	1.11	0.57	0.33	4.22	4.26	0.04	−0.09	0.98	0.64	0.13	0.20
14–19	55.6	14.20	12.16	1.20	0.84	1.97	1.09	0.55	0.33	4.10	4.77	0.06	−0.73	1.08	0.64	0.17	0.28
10–13	56.0	14.19	11.29	1.15	1.75	1.98	1.22	0.54	0.22	4.32	3.88	0.06	0.38	1.02	0.66	0.16	0.20
5– 9	56.7	14.17	11.30	1.08	1.79	1.90	1.20	0.45	0.25	4.42	4.80	0.03	−0.41	1.02	0.67	0.12	0.22
Total gain, 65 days					86.05				16.23				−11.28				17.49
Mean per day		14.05			1.32	1.93			0.27	3.91			−0.17	1.05			0.27

TABLE 1—*continued*

DAYS ANTE-PARTUM	BODY WEIGHT	CALCIUM				MAGNESIUM				SODIUM				POTASSIUM			
		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
65–69	52.0	1.96	0.49	0.55	0.93	0.43	0.14	0.22	0.07	2.92	2.86	0.02	0.04	4.05	4.04	0.35	−0.34
60–64	52.1	1.95	0.48	0.84	0.62	0.39	0.10	0.21	0.08	2.70	2.69	0.01	0.00	4.05	3.83	0.34	0.12
54–59	52.3	1.93	0.51	1.07	0.35	0.40	0.13	0.24	0.03	2.71	2.65	nil	0.06	4.05	3.76	0.30	−0.01
50–53	52.6	1.91	0.44	0.89	0.58	0.39	0.11	0.23	0.05	2.84	2.38	nil	0.46	4.05	3.70	0.35	0.00
45–49	52.8	1.91	0.44	0.61	0.86	0.39	0.06	0.20	0.13	2.75	2.78	nil	−0.03	4.05	3.64	0.31	0.10
40–44	53.0	1.97	0.46	0.80	0.70	0.40	0.12	0.19	0.10	2.71	2.49	nil	0.22	4.05	3.90	0.34	−0.19
35–39	53.5	1.94	0.45	0.86	0.63	0.40	0.14	0.20	0.07	2.75	2.46	nil	0.29	4.05	3.74	0.29	0.02
30–34	54.1	1.98	0.44	0.71	0.84	0.38	0.14	0.18	0.05	2.79	2.88	nil	−0.09	4.05	3.64	0.32	0.09
25–29	54.4	1.98	0.38	0.41	1.18	0.39	0.09	0.14	0.16	2.71	2.48	nil	0.23	4.05	3.50	0.30	0.25
20–24	54.9	1.96	0.41	0.51	1.04	0.38	0.18	0.17	0.02	2.82	2.47	nil	0.36	4.05	3.71	0.34	0.00
14–19	55.6	1.96	0.37	1.04	0.55	0.38	0.17	0.20	0.01	2.79	2.96	nil	−0.17	4.05	3.75	0.36	−0.06
10–13	56.0	1.94	0.31	1.25	0.39	0.39	0.15	0.20	0.04	2.79	2.27	nil	0.52	4.05	3.64	0.34	0.07
5– 9	56.7	1.94	0.26	1.07	0.61	0.35	0.16	0.19	0.03	2.77	2.99	nil	−0.22	4.05	3.06	0.34	0.05
Total gain, 65 days					46.29				4.15				7.26				0.54
Mean per day		1.95			0.71	0.39			0.06	2.77			0.11	4.05			0.01

from the thirty-first to thirty-fifth weeks of pregnancy only thus omitting the final interval to term. The total mean daily retention of nitrogen for the former was 1.74 gm. and that for the latter was 2.24 gm.

Hoffström ('10) was the first to show by means of continuous observations on a woman from the seventeenth week of
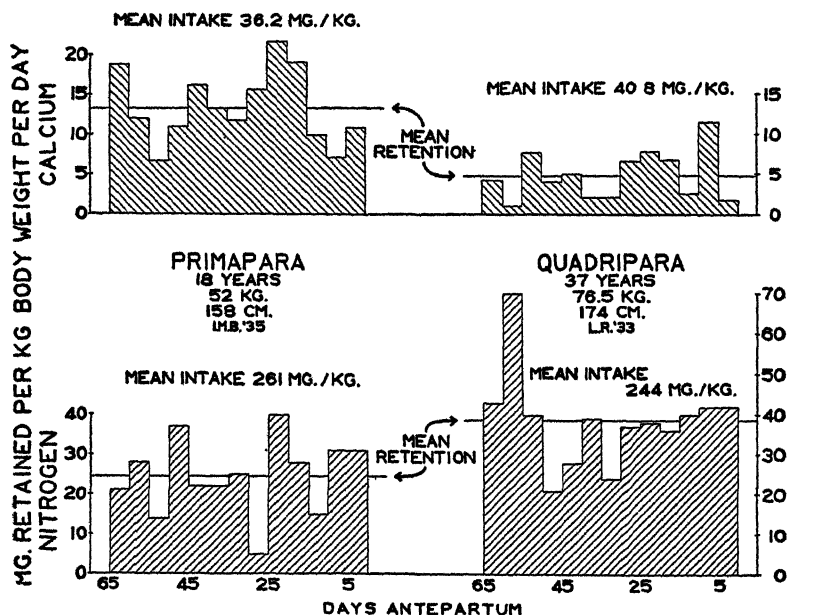
Figure 1 shows the contrast in the continuous daily retentions of calcium and nitrogen per kilogram body weight during 65 days antepartum in two women of widely different backgrounds of nutritional state and physiological constitution, that of the primipara (I.M.B.) being unsatisfactory and that of the quadripara (L.R.) satisfactory.

pregnancy to term that the maternal body acquired a reserve of stored elements beyond the estimated needs of her preparation for puerperium, lactation and the development of the fetus. In accordance with the weight of evidence on the influence of nitrogen retention in pregnancy to milk secretion as shown in the recent review by Garry and Stivens ('36), L.R., who was capable of secreting a large volume of milk beyond

that necessary to nourish her own baby, stored a total of 196.25 gm. of nitrogen during the final 65 days of pregnancy. On the other hand, I.M.B., who developed only an average milk supply, stored but 86.05 gm. of nitrogen, while the case of Hoffström stored 109.8 gm. during the same interval of time. Figure 2 shows that the fetal needs were well covered

ACCUMULATION OF TISSUE MATERIALS DURING LAST 65 DAYS
ANTEPARTUM AS SHOWN BY SUCCESSIVE BALANCE STUDIES
IN THREE WOMEN
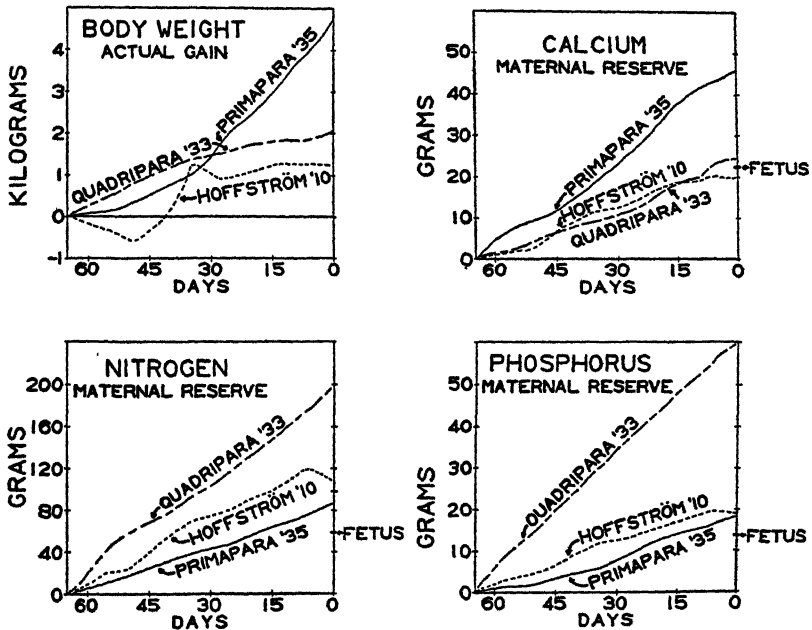
Figure 2 illustrates the accumulation of tissue materials during the last 65 days of gestation as shown by successive balance studies on three women, i.e., Hoffström's ('10) case, the quadripara studied in 1933 and the primapara of the present study as determined by continuous observations during the same interval of time.

by the maternal acquisition of nitrogen during only the last trimester of gestation in all three cases, and that the phosphorus storage was in accord with the nitrogen.

In marked contrast to the results on nitrogen storage are these on calcium (table 1 and fig. 1). With an intake of 32.2

mg. of calcium per kilogram body weight per day, I.M.B. retained 13.2 mg., whereas with an intake of 40.8 mg., L.R. stored on the average only 4.8 mg. daily per kilogram body weight. The total daily retention of 0.71 gm. of calcium per day by I.M.B. exceeded by far the 0.38 gm. by L.R., 0.32 gm. by the woman reported by Hoffström ('10) and 0.28 gm. by the one recorded by Coons and Coons ('35) and falls within the wide variations found by Pyle and Huff ('36) on 24-hour sampling balances.

More pertinent to the knowledge of the relationship of the nutritive state in human pregnancy is the cumulative storage trend of elements seen in the continuous balance studies throughout one reproductive cycle in dogs (Toverud and Toverud, '31) and throughout several cycles in rats (Goss and Schmidt, '30) and dairy cows (Ellenberger, Newlander and Jones, '32). These data particularly for calcium and phosphorus show the influence of individual nutritive conditions on the storage during one complete cycle, i.e., pregnancy, lactation, and reproductive rest, or more; the ability of the animal body to accelerate retention after depletion of reserves; as well as the possibility of increasing body stores through elevation of minerals in the diet so that no drain is imposed upon the maternal organism by reproduction.

The large accumulation of calcium by the primapara (I.M.B.) of this investigation is a challenging observation since she laid down 46.3 gm. in the period of 65 days antepartum as contrasted with 24.7 gm. by L.R. and 20.0 gm. by the subject of Hoffström. It is evident that the three women were under considerably different physiological influences in addition to gravidity (fig. 2) for the former acquired more than twice the actual calcium needs of the fetus during the final thirteen 5-day periods of pregnancy, whereas the other two cases had just satisfied the average fetal requirements during the same interval.

The total accumulation for the nutritive processes of the adjusting maternal and growing fetal bodies is a matter of greatest importance since it may include

the filling in of the nutritive reserves that perchance were existent through a previous state of undernutrition and in some cases upon the growth of the maternal body itself (Hunscher, Hummel, Erickson and Macy, '35).

The dire results of extremely calcium impoverished women are seen in osteomalacia. Maxwell and co-workers ('25, '35) along with Miles and Feng ('25) have studied the etiology, the metabolism and treatment of this deficiency disease, as it is intensified by reproductive demands. They have shown that the bones of adolescent girls may be impoverished by lack of calcium, of vitamin D, or both, and that severe crippling results during reproduction and particularly with repeated cycles. Not only the maternal bones are drained upon but the fetus, as resistant as it is in intra-uterine development to nutritive inadequacies, shows evidence of rickets and osteoporosis of the bones and gross hypoplasia of the enamel of the teeth (Wolfe, '35; Maxwell, '35). Thus in human pregnancy, as in animal (Davidson, '30), the young may start life with a reduced store of calcium. In such a case the individual is forced to carry a greater metabolic load than it is physiologically capable of managing, consequently a crisis arises unless measures are taken to replete the overtaxed and undernourished tissues by a richer diet.

Further evidence in regard to body stores of calcium has been given in the studies of Sherman and Booher ('31) showing the parallel effect of the level of calcium intake on the bodies of rats. These workers have shown that from external appearances the rats may be apparently well nourished but in reality their bodies may be seriously calcium-poor.

Although there was no measurable maternal growth in stature as shown by height and x-ray of the carpal centers[4] in the subject of this investigation, the large maternal storage seems to point to a filling in of bodily stores, in addition to satisfying fetal requirements when the intake had been raised

[4] The x-ray interpretations for growth were graciously made by Lawrence Reynolds, M.D., roentgenologist of Detroit, and T. Wingate Todd, M.D., of the Brush Foundation of Cleveland.

to a higher level than had previously been maintained. It is possible that as the maternal body itself reached saturation during adequate intake, the retentions would become less high as has been shown by Fairbanks and Mitchell ('36) in rats, by Ellenberger, Newlander and Jones ('32) in cows, by Boldt, Brahm and Andresen ('29) in infants, and by Wang, Kern and Kaucher ('29) in children. The need for knowledge of the nutritional history of the animal with an insight into the state of the body stores was expressed by Hart, Steenbock and Hoppert ('23–'24) when they were using dairy cows in experimental inquiry of maintaining calcium and phosphorus reserves during lactation by the metabolic balance method. Certainly the results recorded herein afford further significant information on women during the reproductive cycle.

Trends in the utilization for the other elements stored by I.M.B. are given in table 1 and show that with a mean daily intake of 0.39, 2.77, 4.05 gm. of magnesium, sodium and potassium, the simultaneous retentions were 0.06, 0.11 and 0.01 gm., respectively. In a like manner, a mean daily intake of 1.93, 3.91 and 1.05 gm. of phosphorus, chlorine and sulfur gave a storage of 0.27, — 0.17 and 0.27 gm., respectively. These data are comparable to the other studies in the literature (Hoffström, '10; Coons, Coons and Schiefelbusch, '34; Hummel and co-workers, '36) for magnesium, but lower than those found for the quadripara (L.R.) for sodium and potassium. Furthermore the total daily phosphorus storage was 0.27, 0.30, 0.21 and 0.32 gm. and therefore similar respectively for the cases referred to above. There was an average loss of chlorine by I.M.B. whereas L.R. retained 0.69 gm. daily. Erratic behavior of sodium, potassium and chlorine has been observed by Clark ('26) and Wiley, Wiley and Waller ('33).[5]

The relationship of intake to retention has been the point of interest in most metabolic balance studies for the purpose of establishing fitting dietary standards. There is a wide scatter of retention values in pregnancy on any given level

of intake for both nitrogen and calcium as illustrated in figure 3. This is true not only in isolated balances taken from the literature but also in the present continuous study where the conditions of living and diet were approximately constant.

In summary, the metabolic balance method for determining food standards is not precise and cannot be interpreted too

RELATIONSHIP OF INTAKE TO RETENTION IN PREGNANCY
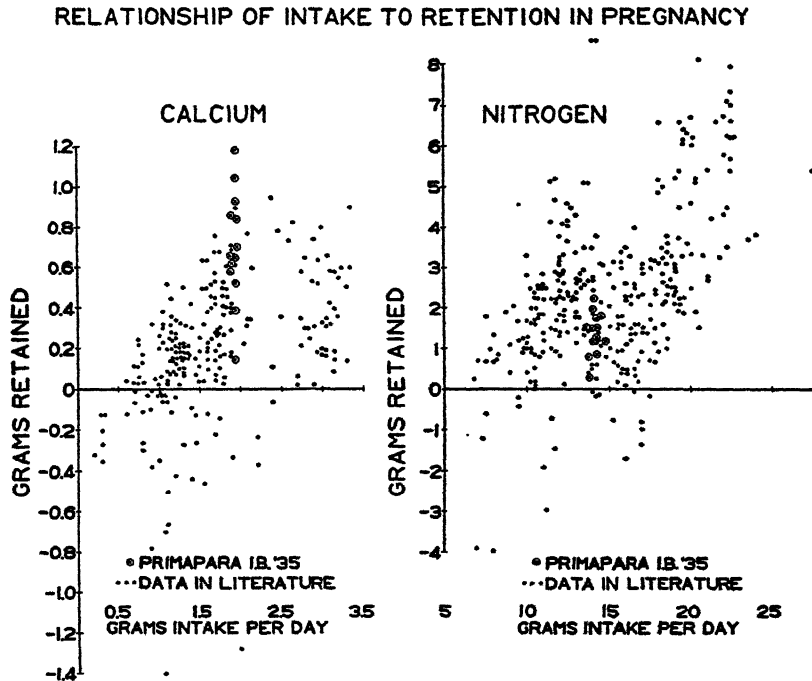
Figure 3 shows the relationship of the level of nitrogen and calcium intake to the amount retained. The results of the present continuous observation in pregnancy are superimposed upon the studies taken from the literature and show not only a wide range of retentions of different individuals on a given level of intake but also significant variations of one individual at different times.

freely without further knowledge of the influencing factors because of such wide physiological variations observed among individuals with highly different nutritive and hereditary backgrounds and of significant fluctuations of the same individual from time to time when maintained under highly controlled environmental conditions and a constant diet.

SUMMARY

1. The present report records the metabolic balances of an 18-year-old primapara observed uninterruptedly during the final 65 days of gestation. Her medical history showed an unsatisfactory nutritional background for 6 preceding years. During the metabolic study she was maintained under highly controlled conditions of living and a constant diet. The results are compared with similar ones on two healthy women during the same interval of reproduction.

2. The mean daily storage of nitrogen, phosphorus, chlorine, sulfur, calcium, magnesium, sodium and potassium was 1.32, 0.27, — 0.17, 0.27, 0.71, 0.06, 0.11, 0.01 gm., respectively.

3. The influence of nutritive state and physiological conditions other than gravidity upon maternal storage of elements in pregnancy is illustrated by the conspicuous differences in total accumulation of nitrogen and calcium by two women of contrasting nutritional and physiological background studied in this laboratory. The total nitrogen and calcium acquired during the same interval of time was 86.05 gm. as contrasted with 196.25 gm. and contrariwise, 46.29 gm. vs. 24.7 gm. for the women with unsatisfactory and satisfactory nutritional histories, respectively.

4. Uninterrupted metabolic balance studies reveal significant variations among individuals under similar circumstances. The results point to the fact that in practice, maternal nutritive state and physiological constitution are important and should be considered when interpreting dietary requirements for pregnancy.

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THE DETERMINATION OF VITAMINS B AND G IN HUMAN URINE BY THE RAT-GROWTH METHOD [1]

O. M. HELMER

The Lilly Laboratory for Clinical Research, Indianapolis City Hospital and Department of Medicine, Indiana University School of Medicine

ONE FIGURE

Recently interest has been renewed in the quantitative determinations of the B vitamins in human urine. Helmer ('35) reported the presence of vitamins B and G in human urine by the rat-growth method. Harris and Leong ('36) have made extensive studies on the content of vitamin B in human urine in relation to intake. Roscoe ('36) has also presented data on the vitamin $B(B_1)$ and $G(B_2)$ content of human urine, and Emmerie ('36) has determined the flavin content of the urine. The earlier literature has been reviewed by Harris and Leong, and Roscoe.

In our preliminary experiments (Helmer, '35) the whole 24-hour urine sample was concentrated to a thick syrup by vacuum distillation at a low temperature and dried in vacuo over sulfuric acid. The dried and powdered urine was mixed with one part by weight of sucrose and one-half part by weight of hydrogenated vegetable oil[2] and fed in quantities equivalent to one-twenty-fifth of the daily 24-hour urine output to rats on a basal diet. These experiments were not wholly satisfactory because some of the animals would not eat all of the urine supplement. Furthermore, the concentration of the urine by vacuum distillation at a low temperature was a time-consuming procedure. In this communication we wish to report a simplified method of preparing the urine supplements

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[1] A report of this work was presented before the American Society of Biological Chemists at Washington, D. C., March, 1936. J. Biol. Chem., vol. 114, p. 48.

[2] Crisco.

by means of adsorption of the vitamins B and G. Harris and Leong ('36) have used a similar technic.

<center>EXPERIMENTAL [a]</center>

The 24-hour urine specimens were adjusted to pH 4.0 by the addition of concentrated hydrochloric acid and then 5 gm. of Lloyd's reagent were added. The urine was stirred for from 15 to 30 minutes with a mechanical stirrer or was allowed to stand in the ice box for 3 or 4 days with occasional shaking. The Lloyd's reagent adsorbate was collected by means of vacuum filtration on hardened filter paper, washed with water, and dried in vacuo over sulphuric acid. As shown in table 2, the 5 gm. of Lloyd's reagent was sufficient to adsorb all of the vitamins B and G from the 24-hour urine sample, inasmuch as a second adsorption with Lloyd's reagent failed to recover any additional quantity of these vitamins.

In the light of the multiple nature of the vitamin $G(B_2)$ complex—György ('35), Birch, György and Harris ('35), Elvehjem and Koehn ('35), Block and Hubbell ('35), Hogan and Richardson ('35), Lepkovsky, Jukes and Krause ('36), and others—the nature of the vitamin supplements for the tests for vitamin B and G had to be considered. The vitamin supplement for the tests for B offered no difficulties since autoclaved yeast should supply the whole vitamin G complex. In the tests for vitamin G in the adsorbates, a supplement was required which would supply the other components of the vitamin G complex necessary for the growth of the rats. In the experiments using the whole dried urine (Helmer, '35), a rice polish extract prepared according to Rosedale ('27) was used for the source of vitamin B. This preparation was a fortunate choice, as shown in figure 1 (group 2), for, when a

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[a] In this paper vitamin B refers to the antineuritic vitamin, vitamin G to lactoflavin. The term 'rat factor' may be considered equivalent to 'B₆' or 'rat antiacrodynia factor' of György, Birch, György and Harris, or to the anti-dermatitis factor of Hogan and Richardson, or to the 'third factor' of Block and Hubbell. The term 'chick factor' will refer to the chick anti-pellagra factor B₃ of Elvehjem, P–P (pellagra-preventative) factor, or to the filtrate factor of Lepkovsky and Jukes. By 'the vitamin G complex' is meant the whole of the heat stable fraction of the water-soluble vitamin B complex.

pure vitamin B[4] preparation made by the method of Stuart, Block and Cowgill ('34) was used as a source of vitamin B, a poor growth response was obtained. The substitution of the Rosedale preparation resulted in an increase in growth rate. This rice polish extract evidently contained enough of the 'rat factor' to cause resumption of growth. Birch, György and Harris have shown that rice polishings are a rich source of this factor. It is evidently not precipitated by the lead
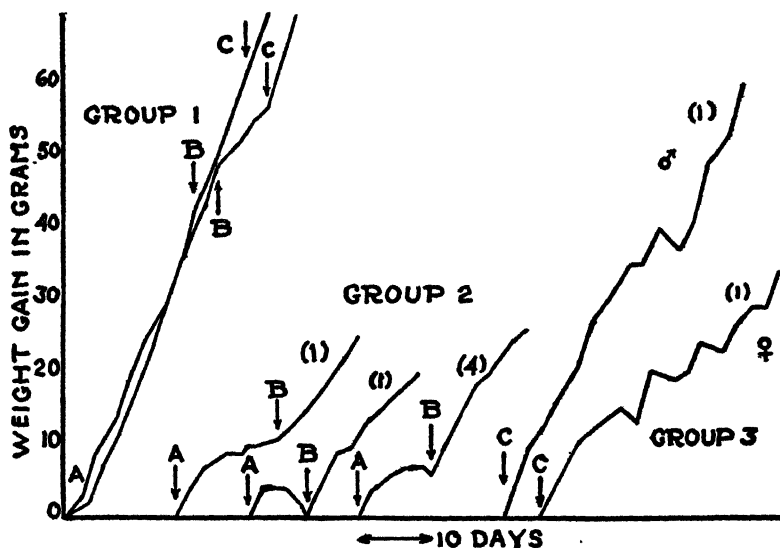
Fig. 1 Growth response of rats on basic diet plus supplements. Group 1, autoclaved yeast plus vitamin B; group 2, Lloyd's reagent adsorbate equivalent to one-twenty-fifth of daily urine output plus vitamin B; group 3, crystalline lactoflavin (0.1 mg.) plus Rosedale's rice polish extract. At A, 1 cc. of a parenteral vitamin B concentrate (diluted 1: 7½); at B, 0.5 cc. of Rosedale's rice polish extract; at C, 1 cc. of Rosedale's rice polish extract.

acetate used in the preparation of this extract. Further evidence is given in figure 1 (group 3) that the rice polish extract of Rosedale's contains the 'rat factor.' Good growth resulted when the basic diet was supplemented with only the Rosedale rice polish extract and crystalline lactoflavin. Figure 1 (group 1) demonstrates that this extract in quantities equivalent to

1 gm. of original rice polishings supplies an adequate amount of vitamin B.

The rice polishing extract was prepared as follows: 500 gm. of rice polishings were extracted with 1% acetic acid by stirring with a mechanical stirrer for 8 hours, the flask being kept at 40°C. After filtering, the extract was concentrated in vacuo at 40°C. to such a volume that 1 cc. was equivalent to 1 gm. of original polishings. Then the extract was treated with lead acetate until no further precipitate could be obtained. When it had been filtered, the lead was removed with H_2S, which in turn was expelled by boiling in vacuo at 40°C. The extract was finally made up so that 1 cc. was equivalent to 1 gm. of polishings. One cubic centimeter of this extract was used as a source of vitamin B. The basal ration used in these experiments contained: vitamin-free casein,[5] 18 parts; cornstarch, 64 parts; hydrogenated vegetable oil,[6] 10 parts; salt mixture no. 185 (McCollum, '18), 4 parts; cod liver oil, 2 parts; and agar, 2 parts.

The rats were kept in separate, double-bottomed, wide mesh cages, and, after a depletion period on the basal diet plus the proper vitamin supplements, the urine adsorbates were given. The Lloyd's reagent adsorbate was placed in glass coasters and a drop of cod liver oil was placed on it, and in some cases sugar was mixed with it to make it more palatable for the rats.

The subjects used in the experiments were medical students and laboratory workers in good health. They were placed on a fixed diet—i.e., one in which the meals were identical in quantity and components three times a day—for a week (see table 1). The urines were collected daily and each 24-hour specimen was treated with Lloyd's reagent, as described above, as soon as each sample was complete. The vitamin B requirement of the subject was calculated by Cowgill's formula ('34). The milligram equivalents (mg.-eq.) excreted in the urine were roughly calculated from the growth data presented in table 2 by first converting to Sherman-Chase units, and then to milligram-equivalents on the basis that one Sherman unit is equivalent to 10 mg.-eq.

[5] The Casein Manufacturing Company of America, Inc., New York.
[6] Crisco.

EXCRETION OF VITAMIN B AND G IN URINE

The growth responses to the urine preparations are shown in table 2, and the relation of the vitamin B excreted to the intake of this vitamin is shown in table 1. The pooled urines of subjects I and II, and the urine of III and of IV contained demonstrable amounts of vitamin B. No significant growth response could be obtained from the urine from subject V when the urine adsorbate was tested for vitamin B. The absence of vitamin B might be due to the fact that so much of the vitamin, calculated by Cowgill's data, was made up of

TABLE 1

The daily caloric and vitamin B intake and vitamin B excretion of normal subjects on a weighed diet

SUBJECT	WEIGHT	HEIGHT	AGE	CALORIC INTAKE	CALCULATED MG.-EQ.[1] INTAKE	CALCULATED MG.-EQ.[1] REQUIREMENT	CALCULATED MG.-EQ.[1] IN EXCESS	MG.-EQ.[1] EXCRETED	PER CENT OF MG.-EQ.[1] INTAKE EXCRETED
	kilos	*cm.*							
I and II	72.6	180	24	2600	7066	5366	1700	1465	21
III	77.2	168	36	2880	7450	6314	1136	1133	15
IVa	76.3	191	19	2600	7022	5706	1360	474	7
IVb	79.5	191	20	3270	9055	7383	1672	1035	10
V	75.0	191	19	3276	8165[2]	6978	1187	±	0

[1] Milligram-equivalents (Cowgill, '34).
[2] 1680 mg.-eq. of total intake were made up of cream.

cream. It is difficult to draw conclusions from these data as to the relation of the amount of vitamin B excreted in the urine to the excess of vitamin B in the diet. However, in three instances the amount of vitamin B in the urine accounts for a considerable proportion of the excess.

The multiple nature of the vitamin G complex makes it difficult at present to make balance studies on vitamin G, since our foodstuffs will have to be reassayed in the light of newer knowledge. The urines from the normal subjects tested so far for vitamins B and G have had in every instance a higher content of vitamin G than of vitamin B.

The urine from subjects I and II, when fed alone as a source of both vitamins B and G, produced a growth response of 6.4

gm. per week. The Lloyd's reagent must have adsorbed some 'rat factor' as well as vitamins B and G. Block and Hubbel ('35) and Lepkovsky et al. ('36) have shown that the 'rat factor' can be adsorbed on fuller's earth. The limiting factor

TABLE 2

The daily excretion of vitamins B and G in the urine of normal subjects

EXPERIMENTAL ANIMAL	SUPPLEMENT	NUMBER OF RATS	DAYS FED	AVERAGE TOTAL GAIN IN WEIGHT	AVERAGE WEEKLY GAIN IN WEIGHT	URINARY VITAMIN B OR G IN SHERMAN UNITS
				gm.	gm.	
+ control	Brewer's yeast	2	35	76	15.0	
+ control	B and autoclaved yeast	3	36	71	12.5	
— control	B	5	42	—1	0.0	
— control	Autoclaved yeast	9	41	4	0.7	
Urine (alone), subject I and II	Urine (1/50)	4	26	24	6.4	
Urine B, subject I and II	Urine (1/50) + autoclaved yeast	5	28	35	8.8	147
Urine G, subject I and II	Urine (1/50 + B	5	28	42	10.5	175
Urine B, subject I and II	Urine (1/25) + autoclaved yeast	3	15	31	13.4	112
Urine G, subject I and II	Urine (1/25) + B	3	15	34	14.4	120
Readsorbed urine B, subject I and II	Urine (1/25) + autoclaved yeast	2	19	—13	—4.8	0
Readsorbed urine G, subject I and II	Urine (1/25) + B	2	20	—4	—1.3	0
Urine B, subject III	Urine (1/50) + autoclaved yeast	2	28	27	6.8	113
Urine B, subject IVa	Urine (1/33) + autoclaved yeast	2	29	18	4.4	47
Urine B, subject IVb	Urine (1/50) + autoclaved yeast	2	28	24	6.2	104
Urine G, subject Vb	Urine (1/50) + autoclaved yeast	2	28	45	11.1	185
Urine G, subject Vb	Urine (1/75) + autoclaved yeast	3	28	28	7.0	175

in the growth response by the urine adsorbates alone may be the 'rat factor' and 'chick factor.' More observations will have to be made with purified supplements to determine this point.

DISCUSSION

The results in the experiments reported in this paper agree with those of Harris and Leong ('36) using 'acid clay' for the adsorption of vitamin B and the 'bradycardia' method of Harris for determining the vitamin B. His method has the advantage over the 'rat-growth' method of requiring less urine for the test. The concentration of the vitamins B and G by means of adsorption methods has the advantage over the vacuum distillation methods in that larger amounts of urine can be given without the toxic effects that Roscoe ('36) described in her experiments with the urine concentrated by vacuum distillation. For example, better growth was obtained in the experiments reported in this paper with one-fiftieth of the daily urinary output than was obtained in those with one-twenty-fifth of the daily output using the urine concentrated by vacuum distillation (Helmer, '35).

Harris and Leong ('36) reported that 5 to 8% of the vitamin B ingested was excreted in the urine. Considering the differences in method, most of the figures reported here are not in great variance with those of the above authors. In subjects I and II, III and IVb the vitamin B excreted in the urine agrees quite well with the calculated excess of the vitamin. A greater discrepancy occurred with subject IV with diet IVa. He is a very active individual, and the diet of 2600 calories did not satisfy his appetite. The additional caloric intake of diet Vb satisfied his appetite, and, although the calculated milligram-equivalent excess of vitamin B was not greatly increased, considerably more of the excess was excreted in the urine.

The results in this paper also agree with those reported earlier by Helmer ('35) and with those of Roscoe ('36) and Emmerie ('36) that vitamin G or flavin is excreted in normal urine.

SUMMARY

The vitamins B and G of normal human urine have been
adsorbed on Lloyd's reagent and tested for activity by the
rat-growth method. When an excess of vitamin B, calculated
by Cowgill's formula, was present in the diet, vitamin B could
be detected in definite quantities in the urine. Vitamin G
was present in the normal urine tested in larger amounts
than vitamin B.

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THE INFLUENCE OF CARBOHYDRATE ON NITRO-GEN METABOLISM IN THE NORMAL NUTRITIONAL STATE

P. S. LARSON AND I. L. CHAIKOFF

Division of Physiology, University of California Medical School, Berkeley

It is now well known that in certain abnormal nutritional states the amount of carbohydrate ingested conditions the extent of protein catabolism. Thus, during fasting and specific nitrogen hunger, carbohydrate prevents the breakdown of body protein (Lusk, '28 a; Landergren, '03; Murlin, '07; Cathcart, '09). More striking, however, is the relation of carbohydrate to the excessive protein metabolism of the phlorhizinized dog. In such an animal, glucose spares protein despite the fact that the glucose producing this effect is being excreted (Ringer, 12; Deuel and Chambers, '25; Gaebler and Murlin, '25). Although the mechanism whereby carbohydrate spares protein is still obscure, this experiment leaves little doubt that it is not always due to an isodynamic replacement of the carbohydrate for the protein that fails to be metabolized. The value of carbohydrate as an inhibitor of still another form of protein destruction, namely, that found in febrile conditions and in hyperthyroidism, has been demonstrated. In both conditions nitrogen equilibrium can be obtained by the use of high calorie diets containing large amounts of carbohydrates (Shaffer, '08; Shaffer and Coleman, '09; Kendall, '29).

The influence of carbohydrate in apparently normal nutritional states was recognized as early as 1856 by Hoppe who showed that carbohydrate lowered the nitrogen excretion in

dogs maintained on an exclusively meat diet. This observation was later confirmed by Lusk (1890) among others.

In the present investigation normal dogs were brought into nitrogen equilibrium by being fed the synthetic mixture devised by Cowgill ('23). By this procedure an equilibrium was established in which the 24-hour nitrogen excretion over long periods of time rarely varied by more than 0.30 gm. The maintenance of this degree of constancy provided an experimental animal for the detection of unusually small changes in nitrogen elimination. At various intervals following the ingestion of the meal, additional quantities of carbohydrate were fed. It was found that the occurrence as well as the degree of nitrogen storage effected by single feedings of carbohydrate was related to the time that elapsed between the consumption of the daily meal and the ingestion of the extra carbohydrate. In the normal nutritional state, carbohydrate does not spare protein unless fed at certain intervals relative to the ingestion of protein. The present study thus establishes a new factor, namely one of time, in the protein-sparing action of carbohydrate under the conditions of nitrogen equilibrium.

EXPERIMENTAL

Care of animals. Adult female dogs were used. They were kept in metal metabolism cages and fed once per day a synthetic diet devised by Cowgill ('23). The casein, sucrose, vitavose, bone ash and Karr's salt mixture ('20) were thoroughly mixed in quantities sufficient for several complete experiments. The uniformity of the mixture was tested by nitrogen determinations in various parts. Fat consisting of 17 gm. of lard and 9 gm. of unsalted butter per 100 gm. of the above mixture was added at each time of feeding. Each dog, receiving about 60 calories per kilogram of body weight, maintained a good appetite throughout the experiment, ingesting within a few minutes all food served. By this procedure, the animals were brought into nitrogen equilibrium after 10 days or 2 weeks of feeding; after this period the

24-hour excretion of nitrogen rarely varied by more than 0.30 gm. of nitrogen. Four days or more of an equilibrium in which the daily excretion of nitrogen did not vary by more than 0.20 gm. were accepted as a suitable control period prior to the feeding of the extra carbohydrate. No nitrogen observations were made near to or during the period of estrus.

The dogs were fed once per day, always in the morning, and catheterized just before the meal was served. In the animals that were subjected to catheterization twice daily, the second was carried out in the evening, exactly 12 hours after the first. Care was taken to maintain environmental conditions (such as temperature of the room, times of feedings and catheterizations) as constant as possible throughout each period of observation.

Methods. The following analytical procedures for urine were employed: ammonia, Folin and Bell method ('17); urea, Van Slyke method ('32); total nitrogen, Kjeldahl method; inorganic sulfur, Rosenheim and Drummond method ('14).

Feces were marked by means of carmine and stored in concentrated sulfuric acid. Its nitrogen content was determined by a modification of the alternative procedure described by Peters and Van Slyke ('32).

I.   The effect produced on the 24-hour nitrogen excretion by a single ingestion of 50 extra gm. of glucose with the meal and at various intervals before and after the meal

The three dogs recorded in tables 1, 2 and 3 were catheterized once daily and shortly thereafter (15 to 25 minutes) fed the regular daily meal. The term '24-hour period' refers to the interval between two catheterizations, and it should be noted that the daily meal was ingested at the beginning of each 24-hour period. Since in each dog the time of the ingestion of the daily meal was kept constant throughout each period of observation, the time of the administration of the 50 extra gm. of carbohydrate has been expressed as time before the next or after the last meal. Extra glucose was administered at the following intervals: 1) With the meal.

2) At 1, 2, 3, 4 and 10 hours after the last meal. 3) At 1, 2, 4 and 6 hours before the next meal (i.e., 23, 22, 20 and 18 hours, respectively, after the last meal).

Each change in nitrogen excretion—when it occurred in response to the carbohydrate—lasted in most cases for 48 hours and consisted of two distinct phases: 1) A drop below the previously established equilibrium level; this was observed in the first 24-hour period. 2) A rise to a level above that of the resting equilibrium; this was found in the following 24-hour period.

With the meal. The response of urinary nitrogen to the administration of 50 gm. of glucose along with the daily meal was tested in three dogs. In dog O (table 1) this led to a drop from a resting equilibrium level of 5.21–5.37 to 4.70 gm. in the 24-hour period in which the carbohydrate was ingested. In the following 24-hour period total nitrogen rose to 5.67 gm. In the third 24-hour period the nitrogen excretion had returned to the resting level. These changes in total nitrogen generally reflect changes in urea excretion. Dogs B (table 2) and T (table 3) showed similar responses.

One hour after the last meal. A single observation on the administration of extra carbohydrate at this interval was made in dog O. There was again observed the decrease in nitrogen excretion in the 24-hour period in which the carbohydrate was ingested, and this was followed in the next 24 hours by the compensatory rise to a level in excess of the previously established equilibrium.

Two hours after the meal. While both phases of the action of carbohydrate on nitrogen excretion were found in dogs B and O when the extra glucose was fed at this interval, the extent of the initial (first 24-hour period) depression in nitrogen output was not so great as that found when carbohydrate was fed either with or at earlier intervals after the meal. Thus, drops to the extent of 0.50 (dog B) and 0.60 gm. (dog O) followed the ingestion of 50 extra gm. of glucose with the meal, whereas the ingestion of the same amount of carbohydrate 2 hours later led to decreases in nitrogen excretion to the extent of 0.20 and 0.35 gm., respectively.

Three hours after the meal. Both the initial fall and compensatory rise in nitrogen excretion were again observed when carbohydrate was fed at this interval (table 1), but the effects were not so marked as those observed when carbohydrate was fed at the first two intervals recorded above.

TABLE 1

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog O

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	*gm.*	*gm.*
1		4.30	0.554	5.33
2		4.37	0.542	5.37
3		4.28	0.528	5.24
4		4.25	0.571	5.28
5	10 hours after meal	4.33	0.516	5.26
6		4.22	0.539	5.21
7		4.36	0.547	5.34
8	With meal	3.88	0.501	4.70
9		4.65	0.551	5.67
10 .		4.34	0.531	5.31
11		4.28	0.584	5.32
12		4.35	0.521	5.35
13	1 hour after meal	3.77	0.561	4.78
14		4.67	0.552	5.69
15		4.54	0.507	5.50
16		4.32	0.555	5.30
17	2 hours after meal	3.98	0.488	4.95
18		4.73	0.541	5.73
19		4.27	0.573	5.27
20		4.40	0.533	5.35
21		4.43	0.556	5.40
22	3 hours after meal	4.16	0.509	5.11
23		4.62	0.527	5.63
24		4.51	0.542	5.48
25		4.38	0.518	5.39
26	4 hours after meal	4.23	0.526	5.23
27		4.30	0.502	5.25
28		4.35	0.534	5.36

Catheterized at 7.45 A.M. daily; fed at 8.00 A.M. daily.

Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter.

Diet nitrogen = 5.54 gm./100 gm. of diet mixture.

This dog maintained a constant weight of 9.6 kg. throughout the experiment.

Four hours after the last meal. Dog O failed to show a definitely measurable response in the nitrogen excretion to the ingestion of 50 gm. of glucose at this time. Although a slight drop may have occurred in dog B, it should be noted

TABLE 2

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog B

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	gm.	gm.
1		5.45	0.505	6.53
2		5.40	0.500	6.47
3		5.34	0.493	6.42
4		5.32	0.477	6.40
5	4 hours after meal	5.18	0.438	6.19
6		5.38	0.525	6.48
7		5.26	0.527	6.39
8	With meal	4.86	0.510	5.88
9		5.88	0.502	6.98
10		5.30	0.534	6.40
11		5.33	0.498	6.39
12	10 hours after meal	5.41	0.537	6.56
13		5.26	0.501	6.34
14		5.32	0.492	6.37
15	2 hours after meal	5.11	0.514	6.16
16		5.59	0.531	6.70
17		5.30	0.522	6.39
18		5.41	0.540	6.53
19		5.40	0.497	6.46
20	4 hours before meal[1]	4.84	0.504	5.90
21		5.35	0.489	6.43
22		5.39	0.553	6.54

Catheterized at 8.35 A.M. daily; fed at 9.00 A.M. daily.
Diet: 125 gm. of diet mixture plus 22 gm. of lard plus 11 gm. of unsalted butter.
Diet nitrogen = 5.66 gm./100 gm. of diet mixture.
This dog maintained a constant weight of 11.5 kg. throughout the experiment.
[1] Twenty hours after the meal fed on day 19.

that a measureable compensatory rise was not observed in the following 24 hours.

Ten hours after the last meal. Dogs B, O and T showed no effect whatsoever of the ingestion of the extra carbohydrate at this interval. Thus in dog O, the equilibrium level was

TABLE 3

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog T

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	gm.	gm.
1		4.56	0.392	5.43
2		4.53	0.402	5.45
3		4.41	0.388	5.30
4		4.47	0.420	5.36
5		4.80	0.407	5.71
6		4.43	0.452	5.34
7	10 hours after meal	4.59	0.397	5.44
8		4.45	0.421	5.36
9		4.40	0.436	5.34
10		4.48	0.413	5.38
11	With meal	3.94	0.417	4.82
12		5.01	0.392	5.93
13		4.62	0.403	5.49
14		4.51	0.416	5.38
15		4.60	0.431	5.52
16		4.56	0.417	5.42
17	1 hour before meal[1]	4.14	0.436	5.06
18		4.90	0.432	5.85
19		4.72	0.418	5.60
20		4.58	0.401	5.48
21		4.52	0.436	5.49
22	2 hours before meal[2]	4.12	0.413	5.00
23		4.58	0.402	5.45
24		4.48	0.413	5.36
25		4.58	0.422	5.50
26	4 hours before meal[3]	4.24	0.435	5.14
27		4.82	0.424	5.66
28		4.61	0.403	5.49
29		4.49	0.430	5.41
30		4.60	0.422	5.47
31		4.47	0.416	5.37
32	6 hours before meal[4]	4.40	0.428	5.36
33		4.58	0.419	5.46

Catheterized at 7.45 A.M. daily; fed at 8.00 A.M. daily.

Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter.

Diet nitrogen = 5.45 gm./100 gm. of diet mixture.

Weight of this dog on first and last day of experiment was 10.2 kg. and 10.3 kg., respectively.

[1] Twenty-three hours after the meal fed on day 16.

[2] Twenty-two hours after the meal fed on day 21.

[3] Twenty hours after the meal fed on day 25.

[4] Eighteen hours after the meal fed on day 31.

5.24–5.37 gm. before glucose administration, while on the day during and the day following the ingestion of carbohydrate the 24-hour excretions of nitrogen were respectively 5.26 and 5.21 gm.

Eighteen hours after the last meal or 6 hours before the next meal. As in the case of the 10-hour interval, carbohydrate had no effect on nitrogen excretion when its ingestion was delayed for 18 hours after the meal (table 3).

Twenty hours after the last meal or 4 hours before the next meal. Although carbohydrate fed at this interval (dogs B and T) influenced the nitrogen excretion, an important difference was observed between the nitrogen spared by early and late feeding of extra carbohydrate. This difference consisted in the time of onset of the change. When the extra carbohydrate was fed 1 to 4 hours after the meal, the initial effect, namely the drop in nitrogen excretion, occurred during the same 24-hour period in which the extra carbohydrate was administered. But when ingested 20 hours after the last meal (i.e., 4 hours before the next meal) the 50 extra gm. of glucose failed to influence the nitrogen excreted during the same 24-hour period in which the extra carbohydrate was ingested, the initial drop occurring in the nitrogen of the following 24 hours. It was the nitrogen to which the next meal contributed that was affected by this late feeding of extra carbohydrate.

This delayed sparing of nitrogen by carbohydrate is more strikingly brought out in the following experiments, in which the time at which the extra carbohydrate was fed more closely approached the ingestion of the next regular meal. These results demonstrate that carbohydrate spares the nitrogen of dogs brought into nitrogen equilibrium only when fed at certain intervals related to the time of the ingestion of the daily meal.

Two hours before the next meal or 22 hours after the last meal. A delayed but nevertheless definite nitrogen response was obtained when extra carbohydrate was fed at this interval (table 3).

One hour before the next meal or 23 hours after the last meal. When fed approximately ¾ hour before the next catheterization, carbohydrate again failed to influence the nitrogen excreted in the 24-hour period in which the extra carbohydrate was ingested (dog T). During the following 24 hours, however, the total nitrogen fell from an equilibrium level of 5.38–5.52 to 5.06 gm.

II. The effect produced by a single feeding of 50 gm. extra glucose at various intervals upon the first and second 12-hour excretions of nitrogen

Since a compensatory rise in the excretion of nitrogen followed in many cases the sparing effect of carbohydrate, it was conceivable that the failure to find a retention of nitrogen in the 24-hour urine sample when carbohydrate was administered at later intervals may have been due to a balance between a decreased and an increased nitrogen output. That this is not the case, however, is shown in the experiments recorded in table 4, in which nitrogen was studied over shorter intervals after carbohydrate administration. Each dog was catheterized at exactly 12-hour intervals. The term 'first 12-hour period' refers to the interval between 7.40 A.M. and 7.40 P.M. (dog L), whereas the 'second 12-hour period' refers to the interval from 7.40 P.M. to 7.40 A.M. Extra glucose was fed at two times: 1) at the same time as the meal, and 2) 12 hours after the meal. The extra carbohydrate was therefore administered at the beginning of both periods in which 12-hour urine samples were collected.

1. With the meal. Although, as already noted, 50 gm. of extra glucose administered at the same time as the meal led to a drop in the urea as well as in the total nitrogen measured in the 24-hour urine sample, nevertheless a decreased nitrogen output does not characterize the nitrogen eliminated throughout the whole of the 24-hour period of observation. A marked drop (0.68 gm. in dog L; 0.91 gm. in dog T; 0.87 gm. in dog S) occurred during the 'first 12-hour period,' at the beginning of which the extra carbohydrate was fed. In the next 12-hour

TABLE 4

The effect of a single feeding of 50 gm. of extra glucose at various intervals upon the first and second 12-hour excretion of nitrogen

	DAY	TIME CARBO-HYDRATE FED	UREA NITROGEN			AMMONIA NITROGEN			TOTAL NITROGEN		
			First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dog L	1		3.49	1.75	5.24	0.275	0.219	0.494	4.03	2.30	6.33
	2		3.54	1.90	5.44	0.218	0.178	0.396	4.03	2.41	6.44
	3		3.38	1.91	5.29	0.260	0.217	0.477	3.91	2.42	6.33
	4		3.35	1.84	5.19	0.234	0.215	0.449	3.98	2.29	6.27
	5	With meal	2.76	2.17	4.93	0.213	0.219	0.432	3.30	2.65	5.95
	6		3.43	2.13	5.56	0.232	0.176	0.408	3.98	2.54	6.52
	7		3.45	1.90	5.35	0.276	0.222	0.498	4.10	2.38	6.48
	8		3.41	1.92	5.33	0.258	0.189	0.447	4.02	2.41	6.43
	9	12 hours after meal	3.46	1.79	5.25	0.255	0.198	0.453	3.98	2.31	6.29
	10		3.37	1.88	5.25	0.241	0.203	0.444	3.91	2.40	6.31
Dog T	1		3.19	1.61	4.80	0.292	0.178	0.470	3.73	2.06	5.79
	2		3.18	1.55	4.73	0.305	0.134	0.439	3.80	1.90	5.70
	3		3.29	1.59	4.88	0.275	0.162	0.437	3.78	1.96	5.74
	4		3.30	1.53	4.83	0.298	0.152	0.450	3.85	1.93	5.78
	5	With meal	2.44	1.80	4.24	0.252	0.167	0.419	2.94	2.21	5.15
	6		3.21	1.87	5.08	0.234	0.167	0.401	3.75	2.23	5.98
	7		3.57	1.54	5.11	0.334	0.125	0.459	4.22	1.82	6.04
	8		3.28	1.60	4.88	0.289	0.155	0.444	3.87	1.96	5.83
	9		3.32	1.55	4.87	0.294	0.153	0.447	3.88	1.91	5.79
	10	12 hours after meal	3.23	1.65	4.88	0.256	0.178	0.434	3.80	2.05	5.85
	11		3.19	1.62	4.81	0.266	0.125	0.391	3.75	1.97	5.72
Dog S	1		2.03	1.34	3.37	0.173	0.173	0.346	2.43	1.71	4.14
	2		2.02	1.35	3.37	0.173	0.176	0.349	2.44	1.69	4.13
	3		2.08	1.25	3.33	0.166	0.175	0.341	2.45	1.61	4.06
	4		2.15	1.28	3.43	0.172	0.177	0.349	2.54	1.66	4.20
	5	With meal	1.32	1.63	2.95	0.144	0.169	0.313	1.67	1.99	3.66
	6		2.17	1.61	3.78	0.165	0.179	0.344	2.55	2.02	4.57
	7		2.08	1.29	3.37	0.162	0.172	0.334	2.48	1.65	4.13

Dog L—This dog was catheterized at 7.40 A.M. and 7.40 P.M. daily; fed at 8.00 A.M. daily. Diet: 125 gm. of diet mixture plus 23 gm. of lard plus 12 gm. of unsalted butter. Diet nitrogen = 5.44 gm./100 gm. of diet mixture. This dog maintained a constant weight of 12.2 kg. throughout the experiment.

Dog T—This dog was catheterized at 7.45 A.M. and 7.45 P.M. daily; fed at 8.00 A.M. daily. Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter. Diet nitrogen = 5.62 gm./100 gm. of diet mixture. This dog maintained a constant weight of 10.3 kg. throughout the experiment.

Dog S—This dog was catheterized at 9.00 A.M. and 9.00 P.M. daily; fed at 9.15 A.M. daily. Diet: 90 gm. of diet mixture plus 16 gm. of lard plus 8 gm. of unsalted butter. Diet nitrogen = 5.64 gm./100 gm. of diet mixture. This dog maintained a constant weight of 9.2 kg. throughout the experiment.

period, however, the nitrogen excreted rose above that excreted in the 'second 12-hour period' of the previous day. Following the retention, the nitrogen excretion of the 'first 12-hour period' returned to the equilibrium level the next day; the nitrogen output of the 'second 12-hour period' remained elevated in all three animals for 2 days as a result of the extra carbohydrate received. The compensatory rise in nitrogen excretion began early after nitrogen retention, and this indicates the inability of the organism to effect permanent storage of spared protein under the conditions of this study.

2. Twelve hours after the meal. No measurable change was observed in the nitrogen metabolism of the 'first' or 'second 12-hour period' when the extra carbohydrate was ingested at the beginning of the 'second 12-hour period.'

III. The effect of continued feeding of extra carbohydrate on nitrogen excretion

1. Twenty-four hour nitrogen excretion. In the preceding sections single feedings of extra carbohydrate were dealt with. In the following experiments (tables 5, 6 and 7) the effects of continued daily additions of extra carbohydrate along with the regular diet were studied. Four dogs were employed, of which dogs T, E and S received the Cowgill diet in the manner described in the early part of this paper; dog Y (table 7) received a high protein diet (over 60% in the form of protein), which was fed at 12-hour intervals.

In all four dogs the ingestion of extra carbohydrate at the same time as the regular meal resulted in a decreased nitrogen elimination in the 24-hour urine sample, and this decreased level was maintained throughout the whole period in which the extra carbohydrate was ingested. The most striking fall in total nitrogen as well as in urea occurred in dog Y, in which the 24-hour nitrogen excretion fell from a resting level of 11.43–11.60 to 10.26 gm. on the second day of carbohydrate administration.

2. *First and second 12-hour nitrogen excretion.* Despite the fact that the 24-hour urine sample showed a nitrogen retention throughout the 4 to 7 days in which the extra carbohydrate was ingested, a lowered nitrogen level did not

TABLE 5

Effect of continued feeding of extra glucose upon nitrogen excretion of dog T

DAY	UREA NITROGEN			AMMONIA NITROGEN			TOTAL NITROGEN			EXTRA GLUCOSE [1]	FECAL NITROGEN	WEIGHT
	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kg.
1	3.26	1.79	5.05	0.304	0.199	0.503	3.83	2.18	6.01	..	Total for	12.2
2	3.18	1.82	5.00	0.286	0.195	0.481	3.76	2.22	5.98	..	first 4	...
3	3.22	1.73	4.95	0.303	0.208	0.511	3.78	2.14	5.92	..	days =	12.2
4	3.20	1.71	4.91	0.278	0.200	0.478	3.76	2.10	5.86	..	1.57	...
5	2.24	2.03	4.27	0.208	0.205	0.413	2.67	2.45	5.12	50	Total for	12.3
6	2.03	2.18	4.21	0.189	0.208	0.397	2.42	2.62	5.04	50	second 4	...
7	2.16	2.12	4.28	0.196	0.204	0.400	2.59	2.51	5.10	50	days =	12.3
8	2.36	2.04	4.40	0.215	0.208	0.423	2.89	2.47	5.36	50	1.59	...
9	2.37	1.95	4.32	0.218	0.203	0.421	2.83	2.37	5.20	50		12.4
10	2.39	2.15	4.54	0.212	0.207	0.419	2.86	2.56	5.42	50		...
11	2.48	2.02	4.50	0.200	0.214	0.414	2.90	2.44	5.34	50		12.5
12										50		
13										50		
14										50		
15	3.71	1.83	5.54	0.284	0.169	0.453	4.24	2.23	6.47	..		12.6
16	3.80	1.76	5.56	0.296	0.202	0.498	4.37	2.18	6.55	..		...
17	3.81	1.71	5.52	0.327	0.191	0.518	4.37	2.12	6.49	..		12.6
18	3.52	1.75	5.27	0.321	0.204	0.525	4.08	2.15	6.23	..	Total for	...
19	3.22	1.79	5.01	0.319	0.194	0.513	3.76	2.17	5.93	..	4 days =	12.6
20	3.31	1.79	5.10	0.331	0.197	0.528	3.89	2.21	6.10	..	1.68	...
21	3.19	1.85	5.04	0.317	0.203	0.520	3.74	2.24	5.98	..		12.6

Catheterized daily at 9.00 A.M. and 9.00 P.M.   Fed at 9.15 A.M. daily.

Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter. Diet nitrogen = 5.77 gm./100 gm. of diet mixture.

[1] Fed with the daily meal.

characterize the nitrogen excretion at all intervals of the 24 hours. In dogs T, E and S the nitrogen output was observed at 12-hour intervals. Although the total nitrogen for the 24-hour period showed a significant drop on all days in which

30 or 50 additional grams of glucose were administered, no measurable decrease was observed during the second 12 hours of the day. It was in the first 12 hours that the whole of the

TABLE 6

Effect of prolonged feeding of glucose on urinary and fecal nitrogen excretion of dogs S and E

| | DAY | UREA NITROGEN | | | AMMONIA NITROGEN | | | TOTAL NITROGEN | | | EXTRA GLUCOSE [1] | FECAL NITROGEN |
		First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours		
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Dog S	1	1.99	1.52	3.51	0.148	0.154	0.302	2.35	1.89	4.24		Total for
	2	1.94	1.50	3.44	0.144	0.177	0.321	2.32	1.88	4.20		first 4
	3	1.96	1.49	3.45	0.150	0.162	0.312	2.30	1.85	4.15		days =
	4	2.01	1.57	3.58	0.147	0.184	0.331	2.38	1.95	4.33		1.56
	5	1.46	1.59	3.05	0.139	0.175	0.314	1.79	1.96	3.75	30	Total for
	6	1.68	1.51	3.19	0.117	0.178	0.295	1.98	1.89	3.87	30	second 4
	7	1.72	1.50	3.22	0.105	0.184	0.289	2.03	1.90	3.93	30	days =
	8	1.67	1.54	3.21	0.103	0.179	0.282	1.99	1.92	3.91	30	1.66
Dog E	1	3.56	2.25	5.81	0.313	0.183	0.496	4.13	2.71	6.84		Total for
	2	3.58	2.20	5.78	0.313	0.208	0.521	4.17	2.64	6.81		first 4
	3	3.52	2.23	5.75	0.319	0.198	0.517	4.08	2.68	6.76		days =
	4	3.57	2.15	5.72	0.292	0.195	0.487	4.16	2.58	6.74		2.04
	5	3.14	2.20	5.34	0.295	0.198	0.493	3.69	2.64	6.33	30	Total for
	6	3.06	2.40	5.46	0.222	0.214	0.436	3.55	2.86	6.41	30	second 4
	7	3.15	2.04	5.19	0.216	0.199	0.415	3.63	2.52	6.15	30	days =
	8	3.36	2.28	5.64	0.218	0.202	0.420	3.83	2.72	6.55	30	1.80

Dog S—Catheterized at 9.00 A.M. and 9.00 P.M. daily; fed at 9.15 A.M. daily. Diet: 90 gm. of diet mixture plus 16 gm. of lard plus 8 gm. of unsalted butter. Diet nitrogen = 5.77 gm. per 100 gm. of diet mixture. Weight of dog 9.7 kg.

Dog E—Catheterized at 9.05 A.M. and 9.05 P.M. daily; fed at 9.15 A.M. daily. Diet: 130 gm. of diet mixture plus 24 gm. of lard plus 12 gm. of unsalted butter. Diet nitrogen = 5.77 gm. per 100 gm. of diet mixture. Weight of dog 13.0 kg.

[1] Fed with the daily meal.

nitrogen retention occurred. Further evidence that the nitrogen retention under the influence of carbohydrate occurs over a short interval was found in dog T, in which the effects of 50 extra gm. of glucose were followed for 7 days. In this dog, although the nitrogen elimination of the first 12 hours

fell from an equilibrium level of 3.76–3.83 (observed prior to the extra carbohydrate feeding) to values between 2.42 and 2.90 gm., a rise instead of a fall was shown in the nitrogen excretion of the second 12 hours.

TABLE 7

Effect of prolonged feeding of carbohydrate on the nitrogen excretion of dog Y on high protein diet

DAY	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN	INORGANIC SULFUR	SUCROSE ADDED
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1	10.00	0.668	11.60	...	
2	9.96	0.810	11.50	...	
3	10.02	0.775	11.44	0.61	
4	10.03	0.785	11.43	0.60	
5	9.44	0.840	11.04	0.56	30[1]
6	8.93	0.752	10.26	0.52	30
7	9.09	0.742	10.51	0.53	30
8	9.42	0.794	10.90	0.54	30
9	9.30	0.794	10.75	0.54	30
10	9.30	0.814	10.75	0.53	30
11	9.57	0.820	11.04	0.56	30
12	10.24	0.870	11.71	0.62	
13	10.64	0.862	12.06	0.63	
14	10.64	0.833	12.07	0.63	
15	10.71	0.901	12.16	0.64	
16	10.58	0.855	12.01	0.63	
17	10.35	0.800	11.73	0.63	
18	9.92	0.787	11.30	0.63	
19	9.80	0.787	11.53	0.61	
20	10.31	0.787	11.82	0.63	
21	10.35	0.782	11.76	0.61	

Weight of dog 7.9 kg.

Diet: casein 59.0%; Cellu-flour 23.5%; cane sugar 11.8%; pancreatin 2.0%; rice bran 2.3%; Karr's salt mixture 1.4%.

Diet nitrogen = 7.77 gm./100 gm. of diet mixture.

Fed 80 gm. of the above mixture at 8.15 A.M. and 8.15 P.M. daily; catheterized at 8.00 A.M. daily.

[1] Fifteen grams sucrose added to diet at each time of feeding.

The cessation of the daily administration of extra carbohydrate led to a most unexpected change in nitrogen excretion. This did not proceed directly from its depressed level to the original equilibrium level established prior to the carbohydrate treatments. In both animals studied (dogs Y and T)

the original nitrogen levels were finally attained after the extra carbohydrate had been withdrawn, but this was preceded by a period in which the nitrogen excreted was definitely in excess of the initial level. For the first 5 days following the removal of the extra carbohydrate, the 24-hour total nitrogen excreted by dog Y (table 7) was markedly above the original equilibrium level (11.43–11.60 gm.), reaching a value of 12.16 on the fourth day. In dog T (table 5) the nitrogen elimination was above the first equilibrium level for 4 days after the extra carbohydrate had been withdrawn. Interestingly enough, this elevation in nitrogen was reflected only in the nitrogen elimination of the first 12 hours, that of the second 12 hours being established at the resting equilibrium level as soon as the extra carbohydrate was removed.

The influence of carbohydrate on the excretion of inorganic sulfur in the urine was determined in dog Y (table 7). Changes in this constituent paralleled those in nitrogen. Thus the administration of the extra sucrose depressed the sulfur excretion from an equilibrium level of 0.61–0.60 to 0.56–0.52 gm. Its excretion remained depressed throughout the whole period in which the extra carbohydrate was ingested, returning to its former level immediately following the interruption of the extra carbohydrate feeding.

In dogs T, E and S, fecal nitrogen was determined at intervals before, during and after the administration of the extra carbohydrate. It should be observed that the extra carbohydrate had no effect on the nitrogen eliminated in the feces.

SUMMARY AND DISCUSSION

From the experimental results presented, some of the limiting factors as well as characteristic features regarding the mechanism whereby carbohydrate spares nitrogen in the normal nutritive state may now be pointed out:

1. The single administration of extra carbohydrate in the normal nutritive state spares nitrogen only when it is ingested at a definite time in relation to the ingestion of protein. When the extra carbohydrate is administered at intervals

far removed from the time of protein feeding, no nitrogen retention occurs even though the amount of extra carbohydrate represents 200 calories or approximately one-third of the daily caloric intake. The interval during which the extra carbohydrate is able to exert a nitrogen-sparing action is limited to 4 hours before and 4 hours after the ingestion of the daily meal. In every case it is the nitrogen to which the protein of the meal contributes that is affected by the extra carbohydrate. This observation thus establishes a new factor, namely, one of time, in the protein-sparing action of carbohydrate in the normal nutritive state. Since, as judged by the nitrogen excretion, the metabolism of protein proceeds most rapidly in the early hours after its ingestion (Lusk, '28 b), this time factor suggests that carbohydrate effectively spares protein only if it is available to the animal at a time when an increase protein metabolism is in progress.

2. Not only the occurrence but also the extent of the nitrogen retention produced by extra carbohydrate administered during the normal nutritive state are determined by the time interval between the ingestion of protein and that of carbohydrate. The most pronounced sparing of nitrogen occurs when the extra carbohydrate is ingested at the same time as the meal or within 1 hour after it. As the interval between the ingestion of these two foodstuffs is lengthened, the nitrogen spared by equal amounts of carbohydrate falls off until finally, when the interval between the two is greater than 4 hours, no sparing whatever occurs.

3. Despite the fact that the 24-hour nitrogen output may show a well-defined sparing of nitrogen in response to a single administration of extra carbohydrate, a decreased nitrogen excretion does not characterize the whole of the 24-hour period during which the carbohydrate is ingested. The interval in which nitrogen retention occurs after a single administration of 50 gm. of extra carbohydrate is very short. In the present study it was observed during the first 12 hours following the carbohydrate feeding. The nitrogen eliminated in the next 12 hours had already shown a rise above the previously established equilibrium level for this period. The nitrogen spared

under the influence of a single feeding of extra carbohydrate is therefore not permanent, its elimination beginning several hours after its storage is effected.

4. Repeated daily additions of extra carbohydrate at the same time that protein is ingested result in nitrogen retention, but this continues only as long as the daily administrations of extra carbohydrate are maintained. The retention of this nitrogen requires the continued presence of the extra carbohydrate, for, although nitrogen continued to be deposited for 7 days under the influence of repeated administrations of extra carbohydrate, the cessation of this extra carbohydrate results in an immediate outpouring of increased amounts of nitrogen, a process that continues for several days after the feeding of extra carbohydrate is discontinued. Here again, despite the fact that the administration of extra carbohydrate was repeated daily for 7 days, actual storage of nitrogen occurred only during the first few hours following the carbohydrate intake, the nitrogen excretion during the later intervals of the 24-hour period being relatively unaffected or even increased. Similarly, when an increased outpouring of nitrogen results from the cessation of daily carbohydrate treatments, this takes place in the interval corresponding to that in which the nitrogen storage is observed (i.e., the first 12 hours), the nitrogen excretion of the later periods remaining relatively unaffected. The fact that an increased nitrogen output in the early hours after protein ingestion was observed for several days after an interruption of extra carbohydrate feeding indicates that the immediate fate of ingested protein is determined in part by the animal's previous carbohydrate intake. This observation is in harmony with the view that nitrogen metabolism in the animal is in a 'continuing' state, as recently pointed out by Borsook and Keighley ('35).

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FURTHER STUDIES CONCERNING THE FORMATION OF THE B-VITAMINS IN THE DIGESTIVE TRACT OF THE RAT [1]

N. B. GUERRANT, R. ADAMS DUTCHER AND RAYMOND A. BROWN

Department of Agricultural and Biological Chemistry, Pennsylvania State College

In previous publications (Guerrant and Dutcher, '34 a, b) it was shown that, under certain experimental conditions, dextrinized cornstarch exhibited unusual dietary properties when compared with sucrose or with the cornstarch from which the dextrinized starch was prepared. The unusual growth responses made by young rats while receiving diets deficient in the vitamin B complex and containing dextrinized cornstarch as the source of carbohydrate have been definitely associated with a characteristic condition existing in the digestive tract of these animals. It has been demonstrated that under such conditions the rat is able to synthesize, in the lower digestive tract, such dietary essentials as are necessary to supplement the vitamin B complex deficient diet. However, in order for the rat to obtain maximum benefits from the products thus synthesized, it is necessary that the animal ingest its excreta, especially the feces.

We have shown (Guerrant, Dutcher and Tomey, '35) that the elaboration of these dietary supplements, by the rat, takes place in the region of the cecum and it was suggested that this elaboration is probably due entirely to the action of certain microorganisms. The marked regularity of the synthesis of these dietary supplements when the rat receives the dex-

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trinized cornstarch diet, and the almost complete lack of such synthesis when comparable diets containing either sucrose or cornstarch are fed, led us to conclude that the above phenomena are due indirectly to differences in rapidity of digestion and subsequent absorption of these carbohydrates. Although it has been frequently observed that, under comparable conditions, greater quantities of dextrinized cornstarch reach the cecum than do either sucrose or unheated starch, much remains to be learned concerning the physiological processes associated with the digestion and the utilization of various forms of carbohydrates. The brief experiments herein reported were carried out with the view of obtaining further information concerning this interesting and important problem.

<p style="text-align:center">EXPERIMENTAL</p>

The investigations of Whipple and Church ('35) have suggested that the marked contrast in the deportment of young rats receiving vitamin.B complex deficient diets containing starch, as compared to other rats receiving comparable diets containing sucrose as the source of carbohydrate, might be due to the small quantity of fatty materials held by the starch particles. Such an explanation might appear to be adequate insofar as accounting for the differences in the nutritive properties of dextrinized starch and sucrose, but is wholly inadequate to account for a similar difference in the nutritive behavior of the dextrinized starch diets and the untreated starch diets, the latter being more comparable to sucrose-containing diets in their nutritive behavior than to the dextrinized starch diets. It does appear, however, that an appreciable quantity of fat in the diet might affect the carbohydrate digestion, thus changing the nature of the micro-flora of the lower intestinal tract, thereby affecting the synthesis of the dietary supplements. In fact it would seem that the inclusion of any substance or substances in the diet which would retard the absorption of the carbohydrate from the digestive tract might be a contributing factor.

In order to study the influence of definite quantities of fat on the synthesis of the dietary substances in the digestive tract of the rat, three diets containing sucrose as the source of carbohydrate were used (diets 369, 370 and 371). As will be observed from table 1, these diets were of similar composition and differed only in that diets 370 and 371 contained 10 and 20%, respectively, of hydrogenated cottonseed oil in place of an equal weight of sucrose.

TABLE 1

Giving the percentage composition of the various diets used in the investigation

DIETS	349	356	369	370	371	372	373	374	375	376
Purified casein	18	18	18	18	18	10	10	18	18	18
Salt mixture	4	4	4	4	4	4	4	4	4	4
CellU flour	2	2	2	2	2	2	2	2	2	2
Cod liver oil	2	2	2	2	2	2	2	2	2	2
Dextrin (autoclaved 4 hours)	74	0	0	0	0	0	0	0	0	0
Cornstarch	0	74	0	0	0	0	0	0	0	0
Sucrose	0	0	74	64	54	0	0	0	0	0
Hydrogenated cottonseed oil	0	0	0	10	20	0	0	0	0	0
White bread	0	0	0	0	0	82	0	0	0	0
Brown bread	0	0	0	0	0	0	82	0	0	0
Corn flakes	0	0	0	0	0	0	0	74	0	0
Dextrin (autoclaved 8 hours)	0	0	0	0	0	0	0	0	74	0
Dextrin (autoclaved 12 hours)	0	0	0	0	0	0	0	0	0	74
	100	100	100	100	100	100	100	100	100	100

Since the inclusion of dextrinized starch (starch which had been moistened with 0.1% solution of citric acid, autoclaved for 4 hours under 15 pounds of pressure and dried) in the diet of the rat had been found to result in a very marked increase in the biological value of the feces, it appeared important to know whether further autoclaving of the starch would bring about more marked changes in this direction. To test this out, four equal portions of cornstarch were weighed out, of which three portions were mixed with equal quantities of the dilute citric acid solution in the usual manner and autoclaved under 15 pounds of pressure for 4, 8 and 12 hours, respectively. The three portions of autoclaved starch

and a fourth portion of untreated starch were placed simultaneously in a large drying oven where they were kept until rendered free from moisture. When dry, each of the four portions was pulverized and incorporated into comparable diets (diets 356, 349, 375 and 376).

The better nutritional deportment observed among those animals which received the dextrinized starch diets in earlier experiments was apparently due to certain changes in the carbohydrate brought about by its treatment with heat. It, therefore, seemed possible that similar changes in the carbohydrate fraction might be brought about in the baking of bread. If such happened to be the case, a well-browned loaf might be expected to exhibit superior nutritional value. In order to investigate this particular problem, forty fresh loaves of bread, purchased on the local market, were used. Each loaf of bread was separated into two portions; the brown crusty exterior and the white spongy interior. The two bread portions were dried at room temperature, pulverized and incorporated into diets 372 and 373 as sources of carbohydrate. In order to have the protein content of these diets comparable to those previously mentioned, the casein content was reduced from 18 to 10%, as will be observed from table 1.

Continuing the reasoning along the same general line, it would appear that a highly dextrinized product such as corn flakes might exhibit very unusual dietary properties when fed to rats in vitamin B complex deficient diets as a source of carbohydrate. A quantity of this product was, therefore, purchased on the local market, dried, pulverized and incorporated in diet 374 in the proportions given in table 1.

In the nutritional studies each of the above described diets was fed to two comparable groups of young rats (six rats per group), in one group of which the animals had access to their own feces and in the other group the animals were denied access to any excreta. All animals were caged in individual metal cages which were provided with coarse screen bottoms. Fresh water and a liberal quantity of the respective

diet were kept before each animal at all times. Body weight changes and food consumption were recorded at weekly intervals. The feces from one group of animals receiving each diet were scraped from the cage pans, placed in a small earthenware container and returned to the cage daily. The feces from the second group of animals were removed from the cage pan daily, the number of pellets counted and then stored in group containers under ether, a different container being used for each group of rats every week. This procedure was followed for 6 weeks for all animals except those which died before the end of this period.

At the end of the experiment, all weekly collections of feces were extracted by shaking successively with four 100 ml. portions of ethyl ether, dried, weighed and their reducing value determined by the A.O.A.C. method ('24) for the determination of starch and other hydrolyzable carbohydrates of feeding stuffs.

Data

Although the experiments were continued for a period of 6 weeks, the mortality among certain groups of animals during the fifth and sixth weeks makes it difficult to compare the responses made by the several groups of animals during the entire experimental period. In order, therefore, to eliminate the factor of mortality from a consideration of the results, only the data obtained during the first 4 weeks of the experimental period are given. These data have been condensed and are given in table 2. The incidence of mortality during the last 2 weeks of the experimental period will be discussed in a subsequent paragraph.

DISCUSSION

It will be observed from the data presented in table 2 that the substitution of 10, or even 20% of hydrogenated cottonseed oil (diets 370 and 371) in the sucrose-containing vitamin B complex deficient diets, for an equal weight of the carbohydrate, resulted in no marked differences in the general

deportment of the experimental animals, except a measurable decrease in the amount of diet consumed. This decrease in food intake was to be expected because of the increased caloric value of the fat-containing diets. It will also be observed

TABLE 2

Showing the average deportment of each of the six animals composing the respective groups during the first 28 days of the experimental period

DIET NO.	GROUP NO.	DIETARY SUPPLEMENT	AVERAGE INITIAL WEIGHT	TOTAL CHANGE IN BODY WEIGHT	TOTAL FOOD INTAKE	DRY ETHER-EXTRACTED FECES				
						Total weight	Per cent	Number of fecal pellets	Average weight of fecal pellets	Reducing value of fecal matter
			gm.	gm.	gm.	gm.			gm.	mg.[1]
369	1	Feces	45	— 3	97	...	...	...	....	...
	2	None	46	—10	84	3.3	3.9	92	0.036	57
370	3	Feces	44	— 2	91	...	...	...	....	...
	4	None	45	—12	78	4.4	5.6	121	0.036	80
371	5	Feces	45	— 4	84	...	...	...	....	...
	6	None	44	—10	69	3.5	5.1	104	0.034	55
356	7	Feces	46	0	82	...	...	...	....	...
	8	None	44	— 7	76	3.3	4.3	103	0.032	43
349	9	Feces	46	22	108	...	...	...	....	...
	10	None	45	— 4	84	7.0	8.3	295	0.024	289
375	11	Feces	46	18	108	...	...	...	....	...
	12	None	46	— 1	90	7.9	8.8	332	0.024	292
376	13	Feces	44	20	107	...	...	...	....	...
	14	None	45	— 3	78	8.4	10.8	353	0.024	327
372	15	Feces	45	94	229	...	...	...	....	...
	16	None	44	90	196	9.4	4.8	287	0.033	230
373	17	Feces	45	73	179	...	...	...	....	...
	18	None	44	78	187	10.0	5.3	269	0.037	280
374	19	Feces	46	11	98	...	...	...	....	...
	20	None	46	0	86	5.8	6.7	220	0.026	138

[1] Reducing values are expressed in milligram equivalents of glucose.

from these data that those animals which had access to their feces (groups 1, 3 and 5) consumed more of the respective diets and lost less in body weight than did the corresponding groups of animals which did not have access to their feces (groups 2, 4 and 6). These relative differences were not

altered by the presence of hydrogenated cottonseed oil in the diet. It does appear, however, that the amount of ether-insoluble fecal matter was increased somewhat as a result of substituting this particular form of fat for an equal weight of sucrose.

It appears, therefore, from these data that young rats receiving vitamin B complex deficient diets which contain sucrose as the source of carbohydrate are benefited somewhat by having access to their own feces during the first week or 10 days of the experimental period. After this time, such feces appeared to have no supplementing value since the test animals declined in weight in a manner similar to that of the rats which did not have access to the excreta. The presence of 10 or even 20% of hydrogenated cottonseed oil in such diets did not alter the results with respect to rate of growth or maintenance of life. Mortalities among the animals receiving the sucrose diets were restricted to groups 2, 4, 5 and 6 and were five, six, two and four animals, respectively, all of which died during the fifth and sixth weeks of the experimental period.

When commercial cornstarch is moistened with a dilute solution of citric acid, autoclaved for 4 hours under 15 pounds of pressure and subsequently dried, some marked changes are brought about in the dietary properties of this substance. This fact may be brought out by comparing the results obtained from animals receiving diets 356 and 349. The general responses of those animals which received the diet containing the untreated starch (diet 356) were comparable to the responses made by animals receiving the sucrose diets, while those animals which received the dextrinized starch diet (diet 349) exhibited a much more favorable nutritional picture. As has been previously reported, those animals which received the dextrinized starch diet and which also had access to their own feces grew at a very favorable rate (22 gm. in 4 weeks), while those animals which received a similar diet, but did not have access to the feces, not only failed to grow but actually lost weight. This loss in weight was not so great, however,

as had resulted among comparable groups of animals which had received the starch or the sucrose diets.

Other noticeable changes, in addition to the increase in growth rate, resulted from the dextrinization of the starch. The percentage of ether-insoluble fecal matter was almost doubled, the number of fecal particles was trebled, the average weight of the fecal particles was reduced approximately one-fourth and the reducing equivalent of the fecal matter was increased sevenfold.

These data indicate quite clearly that dextrinization of cornstarch according to the method described, renders it less readily digested by the normal digestive processes of the rat. Such findings could not have been predicted from our present conception of carbohydrate hydrolysis and metabolism. The complete explanation for the lowered digestibility of the dextrinized cornstarch will have to await further studies. It appears, however, that during the process of dextrinization and subsequent drying, a portion of the carbohydrate fraction is, in reality, dehydrated or caramelized. When ingested by the rat, the caramelized carbohydrate must first become hydrated before it can be acted upon by the hydrolytic agents. This delay in digestion, along with the mild laxative effect of the caramelized products, tends to increase the amount of nutrients reaching the lower bowel and, in consequence, greatly encourages the growth of yeast or similar microorganisms. This appears to be the most probable explanation for the syntheses of the B-vitamins in the digestive tract of rats receiving dextrinized cornstarch diets.

Further autoclaving of the starch for 8 and 12 hours, respectively, did not greatly alter the nutritional properties of the starch beyond that which had been obtained by the 4-hour autoclaving (diets 375 and 376). There did appear to be some increase, however, in the per cent of fecal matter, in the number of fecal pellets and in the amounts of reducing substances in the feces, as a result of the longer periods of autoclaving. These increases were not great. The mortalities among the animals receiving the starch diet or the dextrin-

ized starch diets were restricted to groups 8, 10, 12 and 14 and were three, two, one and one rats, respectively, all of which died during the sixth week of the experiment.

The growth responses obtained as a result of feeding groups of young rats diets containing different parts of the baker's loaf (diets 372 and 373) were not according to expectation. It is highly probable, however, that these results were greatly influenced by the presence of considerable amounts of the B-vitamins in the original bread. All animals receiving the diets containing the bread made very unusual growth responses. In fact, the growth rate was so rapid that any beneficial effect of feces to those animals was obscured. The somewhat suppressed growth rate of those animals (groups 17 and 18) which received the diet containing the outer portion of the loaf (diet 373) indicates that the growth-stimulating value of the loaf had been reduced by the baking process. In all probability this reduced growth rate was due to a partial destruction of the B-vitamins near the exterior of the loaf.

The dietary supplementary value of the feces from those animals which received the diet containing corn flakes (diet 374) was quite definite, but not so marked as had been observed among those animals which had received the diets containing the dextrinized cornstarch diets. From the data obtained from the animals receiving this diet, as well as from those animals receiving the dextrinized starch diets (as contrasting those animals which had received the sucrose and the starch diets), it appears that a relatively large percentage of fecal matter of low density and of high reducing value is associated with the formation of substances in the digestive tract of the rat, which are effective in supplementing a vitamin B complex deficient diet. A large fecal output, composed of numerous pellets of relatively high reducing value, is believed to be indicative of a definite laxative effect in the digestive tract, while few fecal particles of low reducing value might indicate the opposite condition. In the above experiments, however, the average weight of the individual fecal particle

bore little if any relationship to their average size. The fecal pellets from the dextrin-fed rats were invariably large, fluffy and highly aerated, while those from the sucrose-fed rats were somewhat smaller in size and were very compact. The superior physical condition of the digestive tract of the dextrin-fed rats, as contrasting the sucrose-fed rats, was always apparent.

The recent contribution of Bliss and Green ('36) has greatly extended our knowledge concerning the phenomenon of 'refection.' While it is generally considered that refection and those conditions which cause coprophagy to vitiate experimental results are different phenomena, there remains the possibility of some relationship existing between the two conditions. In fact, several factors point toward the existence of such a relationship. While it is entirely possible that the two phenomena are caused by different types of microorganisms, it is also possible that both are caused by the same type of organism but in different parts of the rat's digestive tract. It seems reasonable to suppose that in the case of refection, where there is a direct absorption of the growth-stimulating factors, the elaboration must take place in the upper digestive tract, probably in the small intestines. Since in the other case, it is necessary that the rat ingest the excreta in order to receive full benefit of the products formed, this suggests that the elaboration takes place in the lower gut. The fact remains that in one case the supplementary substances are absorbed directly from the digestive tract, while in the other it is necessary that the animal re-ingest the feces in order to receive full benefit from the products synthesized in the rat.

SUMMARY

Further studies have been made concerning the role of the diet in the formation of the B-vitamins in the digestive tract of the rat. From results obtained during this investigation, the following conclusions are drawn:

1. The presence of 10 or 20% of hydrogenated cottonseed oil in the vitamin B complex deficient diet, containing sucrose as a source of carbohydrate, does not lead to a more favorable production of the B-vitamins in the digestive tract of the rat.

2. Further autoclaving of the starch beyond the usual 4-hour period does not lead to a significant increase in the amount of supplementary substances eliminated by the rat in its feces.

3. Those diets which, when fed to rats, result in relatively large percentages of fecal matter, having a low density and a high reducing equivalent, are conducive to the formation of substances in the digestive tract which are effective in supplementing a vitamin B complex deficient diet.

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THE INFLUENCE OF LACK OF VITAMIN A IN THE DIET ON THE PHAGOCYTOSIS PROMOTING PROPERTIES OF THE BLOOD SERUM[1]

ERNST GELLHORN AND JOAN O. DUNN

Department of Physiology, College of Medicine, University of Illinois, Chicago

In spite of the fact that one of the outstanding symptoms occurring in vitamin A deficiency is xerophthalmia and that not infrequently other infectious processes are observed during the disease, the effect of vitamin A on those immunological processes which play a major role in the defense of the organism against infections are far from being clear. There are only very few investigations dealing directly with the immunological properties of the blood serum under conditions of vitamin A deficiency. Zilva ('19) studied amboceptor and agglutinin reactions in vitamin A deficiency and found them unaltered. Findlay and MacKenzie ('22) investigated the phagocytic index in vitamin A deficiency and found no deviations from the control. Werkman ('23) reported on a phagocytosis study in vitamin A deficiency which was also negative. All these investigations were performed on an insufficient number of animals. Moreover, the significance of the variations in the phagocytic index which did occur is not clear since the variations occurring in controls were not studied. It seems doubtful whether any conclusions can be derived from this experimental material. A reinvestigation of the immunological properties of the blood serum under the conditions of vitamin deficiency and particularly vitamin A defi-

ciency seems to be very important since "a considerable
reduction in resistance to infection occurs in animals suffer-
ing from a marked and prolonged deficiency of the fat soluble
vitamins" (Robertson, '34). It should concern mainly the
process of phagocytosis of which Arkwright and collaborators
('31) say that "the all important fact in relation to immunity
is the development of thermostabile specific antibodies which
promote phagocytosis." Before we accept the idea that the
immunological properties of the serum are unaltered under
the conditions of vitamin A deficiency and that the infections
occurring under these conditions are secondary to the altera-
tions of the epithelium of the skin and mucous membranes
(Mori, '22; Wolback and Howe, '25) an extensive study of
phagocytosis with modern methods seems imperative.

Such an investigation was conducted during the past 2 years
in which, after several hundred control experiments, the
phagocytosis method of Hamburger ('27) yielded results of
a very high degree of accuracy.

METHOD

The experiments were carried out on fifty-seven vitamin A
deficient rats and twenty-two control rats during March and
April, 1935, and January and April, 1936.

Technic of phagocytosis study. The leukocytes were ob-
tained from rats which had been injected with 25 cc. of a
saponin solution 1: 50,000 intraperitoneally. Sixteen hours
later a second intraperitoneal injection of 25 cc. of 0.9% NaCl
was made and 2 hours later the rat was killed and the ab-
dominal cavity was washed with 100 cc. of citrate-sodium
chloride solution. The fluid was filtered through a double
layer of gauze and the number of leukocytes present in 1
cubic millimeter was ascertained. This method yielded a
total of 100,000,000 to 150,000,000 leukocytes. The leukocytes
were centrifuged off and the citrate-sodium chloride solution
was decanted and washed twice with 0.9% sodium chloride.
The experimental animals and the controls were killed and
the blood was obtained from heart or carotid artery and
collected in a centrifuge tube. It was allowed to coagulate

and the serum was used for the experiment. Test tubes fitted with ground glass stoppers were filled with the following solutions.

    0.6 cc. starch suspension in 0.9% sodium chloride[2]
    0.1 cc. m/15 phosphate buffer pH 6.8
    0.1 cc. 0.9% sodium chloride
    0.1 cc. serum diluted 1: 5 with 0.9% sodium chloride
    0.1 cc. leukocyte suspension in sodium chloride

The mixture was shaken and the test tubes closed with the well-greased glass stoppers. They were placed immediately in rotating cylinders in an incubator which was kept at 37°. An apparatus similar to that described by Robertson and collaborators ('24) was used. The tubes were kept rotating in the incubator for from 25 to 45 minutes. Thereafter they were removed, shaken, placed on a rack, and 1 drop of a 10% formalin and 2 drops Lugol solution were added to the solution. Then the percentage of leukocytes which had ingested starch particles was determined in samples from the control and the experimental tubes. More than 200 cells were counted in every tube, but not infrequently twice as many cells were investigated. The accuracy of this improved Hamburger method is evident from the table 1, which shows the phagocytosis promoting power of two control sera in a number of experiments. It indicates that the variations in the phagocytosis promoting properties of two sera obtained from normal rats is generally less than 10% and very frequently even less than 5%.

The rats used for this experiment weighed about 40 gm. each. The diet consisted of:

	%
Casein	18
Salt mixture	4
Fleischmann's irradiated dried yeast	8[3]
Starch	65
Corn oil	5
Total	100

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[2] The starch particles were of a uniform size, approximately half as large as the leukocytes. We had about one particle for each leukocyte in each preparation.

[3] The irradiated yeast contained 10 D = 400 international units per gram. The daily consumption of vitamin D was approximately 15 D or 600 international units.

The salt mixture consisted of:

NaCl	0.173
MgSO$_4$ (anhydrous)	0.266
NaH$_2$PO$_4$(H$_2$O)	0.347
K$_2$HPO$_4$	0.954
CaH$_4$(PO$_4$)$_2$H$_2$O	0.540
Ferric citrate $\frac{1}{2}$ H$_2$O	0.118
Calcium lactate	1.3
	3.698

TABLE 1 [1]

Control experiments. Comparison of the phagocytic index of sera of two normal rats. Phagocytic index

EXPERIMENT NO.	RAT I	RAT II	DIFFERENCE IN PER CENT
1	49.2	49.6	+1
2	63.9	62.2	—2.3
3	37.0	39.3	+6.3
4	29.0	30.7	+6.0
5	45.2	46.7	+3.5
6	60.8	65.1	+7.0
7	65.5	68.9	+5.2
8	63.4	66.5	+5.0
9	65.0	67.7	+4.2
10	66.6	64.1	—3.6
11	38.4	35.6	—7.0
12	47.8	46.6	—2.3

[1] Phagocytes obtained from a rat by the procedure described above were incubated in two different sera (rats I and II) and the percentage of leucocytes which had ingested a starch particle was determined (phagocytic index). Column 4 shows the difference in per cent if the phagocytic index of the serum of rat I is taken as 100.

The differences in the absolute values of the phagocytic indices of the ten experiments of table 1 are due to the fact that the incubation time varied in different experiments between 20 and 40 minutes.

In the case of the controls Smaco, as a source of vitamin A, was added (2.5 cc. to 3333 gm. of food). It contains 7500 U.S.P. units per gram.

RESULTS

Table 2 indicates that the animals were sacrificed for the purpose of the phagocytosis experiment after growth had ceased completely and a distinct loss in weight was established. All experimental animals showed typical signs of

TABLE 2
The phagocytosis experiments, 1935

DATE	INITIAL WEIGHT	MAXIMUM WEIGHT	FINAL WEIGHT	WEIGHT LOSS IN PER CENT	DURATION OF EXPERIMENT IN DAYS	PHAGOCYTOSIS, PER CENT		PHAGOCYTOSIS IN PER CENT OF CONTROL
A. Control rats								
4/11	33.5	135.8	135.8	0	40	356[1]/560[2]	63.5 } 66.2	100
	32.0	151.9	151.9	0		335/482	69.5	
B. Vitamin A free rats								
4/11	37.6	124.0	96.2	22.4		65/200	32.5	39.2
	41.5	134.2	104.2	22.0		125/175	71.2	107.8
	38.3	128.3	92.1	28.2		200/305	65.5	99.0
	36.9	133.0	113.4	14.5	40	120/200	60.0	90.8
	31.3	100.8	79.0	21.5		120/240	50.0	75.6
	35.1	92.5	84.7	8.3		110/235	46.7	70.7
	39.1	140.8	114.5	18.4		93/200	46.5	70.3
	39.0	140.3	122.5	12.5		120/205	58.4	88.4
A. Control rats								
4/13	38.5	148.0	148.0	0	42	130/200	65.0 } 65.2	100
	34.8	139.1	139.1	0		135/207	65.4	
B. Vitamin A free rats								
4/13	38.5	123.2	104.4	15		250/415	60.4	92.6
	38.9	122.5	94.7	22.7	42	123/194	63.2	92.5
	33.0	116.3	104.8	9.7		220/367	60.1	92.4
	36.8	116.5	102.3	11.7		250/325	77.1	118.5
A. Control rats								
4/17	31.5	173	173	0	46	325/570	56.9[1] } 56.9	
	40.5	174	174	0		132/232	56.8	
B. Vitamin A free rats								
4/17	37.7	139	112	19.2		400/595	67.4	118.5
	32.4	110.3	84.6	23.3	46	185/245	75.6	133.0
	33.3	119.6	84.8	29.0		259/464	56.0	98.5
	39.2	126.5	88.6	30.0		185/295	62.8	110.8
A. Control rats								
4/22	39.0	198	198	0	51	320/567	56.5	
B. Vitamin A free rats								
4/22	41.8	122.3	81.3	23.6		5/110	4.6	8.2
	34.0	104.5	68.4	35.0		285/385	74.1	131.8
	37.6	106.0	82.8	21.8		200/350	57.3	101.5
	38.0	129.5	82.2	36.5		142/247	57.6	102
	36.6	125.7	100.3	19.7		251/335	75.1	133.0
A. Control rats								
4/27	32.0	165.8	164.5	0.5	55	58/148	39.2 } 37.5	
	37.0	210.0	210.0	0		128/348	36.9	
B. Vitamin A free rats								
4/27			117.5	91	22.5	55/285	19.3	51.6
			111.4	86.8	22.1	54/164	32.9	87.9
			128.4	90	30	56/256	21.9	58.5
			122.2	86.8	29	37/157	23.6	63.1
			132.2	94.2	29	45/155	29.0 ·	77.5
			112.5	80.5	28.3	43/153	28.2	75.3
			107.8	77.5	28	41/161	25.6	68.4
			118.0	81.8	30.6	30/230	13.1	34.8

[1] Number of leucocytes containing starch particles.   [2] Total number of leucocytes counted.

vitamin A deficiency. Particularly marked was xerophthal-
mia, alterations in the skin around the toes, a cyst at the back
of the tongue, inflammatory processes of the genital organs.
Table 2 presents the results of the first phagocytosis experi-
ment which comprises twenty-nine experimental animals and
eleven control animals. This experiment was carried out
during 5 days after the experimental animals had been on the
vitamin A free diet for from 40 to 55 days. The changes in
phagocytosis are expressed in percentages of the controls.
The variations in the phagocytosis of the controls are ex-
tremely small.

In this group of experiments those animals which showed
the most marked loss in weight were sacrificed first. The
experiments showed that the alterations in phagocytosis were
not uniform. In the first experiment, April 11, 1935, four or
five of the eight experimental animals showed a very marked
drop in the phagocytosis promoting power of the serum. In
the following three experiments we observed either no change
or an increase in the phagocytic index, but in the last experi-
ments phagocytosis was markedly decreased. As will be seen
later, the second experiment carried out in the following year
gave similar results. Obviously the phagocytic index is the
result of at least two opposing factors. It seems to be very
likely that under the influence of the infections which actually
take place in vitamin A deficient rats the phagocytosis pro-
moting properties of the serum are increased just as they
are in any other infections under ordinary conditions, but if
the conditions of vitamin A deficiency continue the ability of
the animal to produce these immune bodies is greatly dis-
turbed. In such cases we find a considerable decrease in
phagocytic index in spite of the fact that these animals have
fully developed signs of vitamin A deficiency and severe in-
flammatory processes in various parts of the body.

In regard to details, it may be seen quite clearly from table
2 that there is no relationship between the weight loss and the
phagocytic index, neither does a parallelism exist between
the severity of the clinical symptoms and the phagocytic index.

In view of the fact that it is to be expected that the resistance of different animals and the development of the active immune bodies in these animals show considerable temporal individual variations, it is easily understandable that the time at which
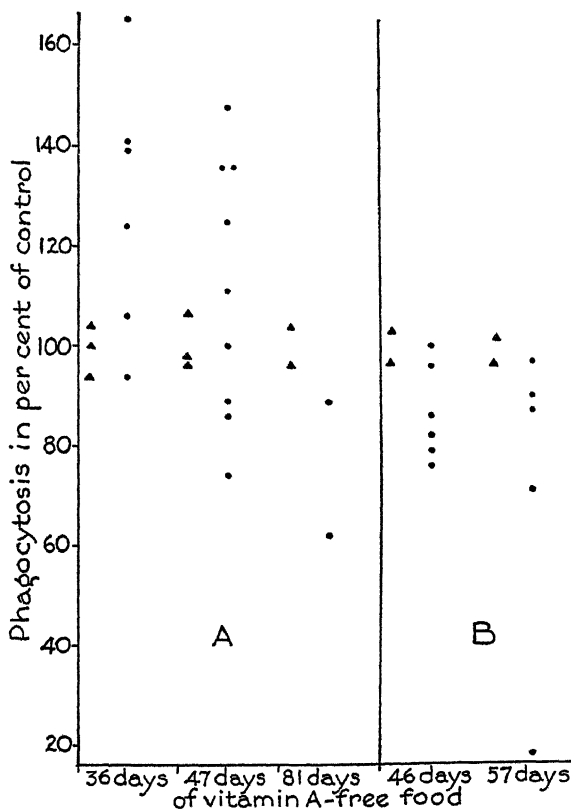
Fig. 1   The influence of a vitamin A free diet on the phagocytic index.   Controls are represented by triangles and the indices of the experimental animals (vitamin A free diet) are represented by dots.   The data shown in A and B were obtained in two different experiments.

a decrease in phagocytosis occurs varies for different experimental animals of the same group.   There seems to be, however, as a rule, the tendency that the decrease in phagocytosis is more marked with increased duration of the vitamin A free period.   This is clearly brought out in the second vitamin

experiment (fig. 1A) in which after 36 days of vitamin A free diet the phagocytic index was either increased or normal, after 47 days five animals showed an increased phagocytic index and three a decrease, and after 81 days the two experimental animals both showed a decrease in phagocytosis. In the third vitamin experiment (fig. 1B) in which the first group of animals was sacrificed after 46 and 57 days, respectively, only decreases in phagocytic index were observed.

The experiments lead to the conclusion that both increased and decreased phagocytic indices are observed in vitamin A deficient rats. The increase is thought to be due to an increased formation of antibodies induced by the infectious processes which occur in vitamin A deficiency. The decrease is believed to be due to an exhaustion of these antibodies or rather to the inability of the body to produce new ones. Thus it may be said that in the earlier part of the vitamin A lack experiments increased phagocytosis occurs in the face of infections as is found under the same circumstances in animals on a normal diet. Obviously, those cells and organs responsible for the production of those substances in the serum which promote phagocytosis and which are present in the serum in increased amounts are less vulnerable than the epithelial cells which show keratinization. Since growth ceases before a decrease in phagocytosis occurs it may also be said that those organs with internal secretions (thyroid, pituitary, thymus) which under the conditions of a normal vitamin diet are responsible for physiological growth are more vulnerable than the systems responsible for the formation of antibodies. This differential susceptibility of various organs in the organism to vitamin A deficiency explains the dispute of various authors on the question whether vitamin A is an anti-infective vitamin or not. The direct experiments which have been carried out to decide this question and which were mentioned in the introduction were mostly negative, but the results are, for reasons given above, unconvincing. The study of the resistance of animals to natural and artificial infections shows quite variable results. This, too, is understandable if one

takes into account the fact that, depending on the general state of health of the animals, the duration of the period of vitamin A deficiency and the mode of infection, the animals may exhaust their defensive forces at different times and to a different degree. We come, therefore, to the conclusion that in vitamin A deficiency of a degree leading to pronounced symptoms (cessation of growth, loss of weight, xerophthalmia, infectious processes on skin and mucous membranes) a normal or even an increased phagocytic index may occur. Upon further progression of the disease, however, a decrease is observed.

The fact that in the majority of cases (table 3) a decreased resistance to natural or artificial infections occurs in vitamin A deficient rats is well explained on the basis of our observations that finally vitamin A deficiency leads to a very marked

TABLE 3

Summary of all vitamin deficiency experiments

Number of animals: 57

11 animals show no change in phagocytic index
16 animals show increased phagocytic index
30 animals show decreased phagocytic index

decrease in the phagocytic index. It seems to be of importance to emphasize that results applicable to various types of bacterial infections can be arrived at on the basis of experiments which do not take the specific properties of the bacteria into account, but which are based on experiments on phagocytosis of an inert material (starch). Obviously we deal here with a factor common to all processes of phagocytosis and the results are not obscured by the specific properties of certain kinds of bacteria.

This discussion makes it clear that we believe that the action of vitamin A on phagocytosis is not a direct one on the leukocytes or the particles ingested, but on those cells and organ systems in the body which, under the influence of hormones and vitamins, produce those phagocytosis promoting properties of the serum which determine the phagocytic index.

In other words, we would not expect the addition of vitamin A to the serum in vitro to increase the phagocytic index. However, the performance of such an experiment is difficult because of the insufficient water solubility of the vitamin A.

If our observations are correct that during the development of a vitamin A deficiency in rats the phagocytic index increases or decreases it is to be expected that control values should be obtained again when such vitamin deficient rats are put on an ordinary diet for a length of time sufficient to cure the animals completely. Such experiments have been carried out with six

TABLE 4

Reversibility of the effects of vitamin A deficiency on phagocytosis

RAT NO.	NUMBER OF DAYS ON VITA-MIN A FREE DIET	NUMBER OF VITA-OF UNITS MIN A IN-JECTED	NORMAL DIET DAYS	WEIGHT AT THE END OF THE VIT-AMIN A FREE PERIOD	WEIGHT AT THE END OF THE SUB-SEQUENT PERIOD WITH NORMAL DIET	PHAGOCYTOSIS		PHAGO-CYTOSIS IN PER CENT OF CON-TROLS
VI, 11	61	1200	51	134	219	150/350	42.9	102.9
VI, 2	66	800	46	152	263	145/355	40.9	98.1
VI, 5	66	800	46	137	196	120/310	38.4	92.2
VI, 7	66	800	46	146	216	135/360	37.6	90.1
V, 5	83	400	29	131	171	245/620	39.6	94.7
V, 9	83	400	29	143	175	278/728	38.2	91.3
Control 1					250	269/628	42.8 } 41.7	100
Control 2					220	290/710	40.8	

rats, each of which showed marked symptoms of vitamin A deficiency at the end of 61 to 83 days of vitamin A free diet. The animals were treated by intraperitoneal injections of vitamin A and our standard diet as described above was given for 51 to 29 days (table 4). They were then apparently normal and were sacrificed and the phagocytosis promoting properties of their sera were determined. The phagocytic indices are nearly all within the limits of the controls, and the two which are below that value show insignificant differences. It may be said, therefore, that the phagocytosis promoting properties of the serum of rats subjected to vitamin A deficiency are altered

in a reversible fashion since a normal diet may restore control values.

We are cognizant of the fact that the infection itself may have been the sole cause of the exhaustion of the phagocytosis promoting properties of the serum. However, inasmuch as the return to normal diet promptly restores the health and the phagocytosis promoting properties of the serum of the animals we feel that vitamin A plays the major role.

Finally the question arises whether weight loss itself may not alter the phagocytosis promoting properties of the serum. This important question will be discussed in a subsequent paper. Suffice it to say here that much greater losses in weight than were observed in our vitamin A deficient animals are necessary in order to cause a decrease in the phagocytic index.

SUMMARY AND CONCLUSIONS

The following results were obtained in experiments carried out with an improved Hamburger method on the phagocytosis promoting properties of the serum under conditions of vitamin A deficiency.

1. Rats which under the influence of vitamin A deficiency developed marked clinical symptoms, such as loss in growth and body weight, xerophthalmia, infections of skin and mucous membranes, may show either increases or decreases in the normal phagocytic index. This is due to the fact that the infectious processes which occur under vitamin A deficiency elicit the production of antibodies to an increased degree during the earlier states of the vitamin A deficiency. Later on the production of new antibodies does not keep pace with the demands of the organism and, as a result, the phagocytic index decreases considerably. Since a fully developed vitamin A deficiency may be accompanied by an increased phagocytic index this vitamin does not seem to be indispensable for the production of antibodies. Upon further progress of the disease, however, the phagocytic index decreases and explains well the fact that such animals are more susceptible to infections than animals under normal diet.

2. Weight loss and severity of symptoms do not run parallel to the changes in phagocytic index.

3. The changes in phagocytic index are completely reversible when the vitamin A deficient animals are put on a normal diet for a sufficiently long period of time.

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PHOTOMETRIC DETERMINATIONS OF UREA, URIC ACID, CREATININE AND HEMOGLOBIN IN THE BLOOD OF SCORBUTIC GUINEA PIGS

SUE POTTER VILTER AND RUTH JOHNSTIN

Chemical Laboratory, Wellesley College, Wellesley, Massachusetts

ONE FIGURE

Two years ago, in connection with a study of the anti-scorbutic properties of galacturonic acid, concurrent analysis of the blood of scorbutic guinea pigs indicated that a more detailed investigation of this phase of the problem might produce data which would be of considerable diagnostic value (Johnstin and Potter, '35). In spite of the vast amount of work which has been done upon scurvy, the mechanism of the pathological progression of this disease is still uncertain. The study is complicated because the symptoms vary with different animals of the same species, and the rapid course of this disease in laboratory animals is quite·different from the slow development in man.

The nitrogen metabolism of scorbutic guinea pigs has been studied by several investigators, including Palladin and Kudrjawzewa ('24), Jarussowa ('26, '28), Nagayama and Sato ('28), Shipp and Zilva ('28) and Tomita ('32), but the conclusions, based chiefly on the results of urine analysis, are not in agreement. It is the opinion of most workers in the field that scurvy is accompanied by deranged nitrogen metabolism and that there is pathological retention of nitrogenous wastes. From a study of the uric acid content of scorbutic urine, Nagayama (Nagayama and Sato, '28) con-

cluded that there was retention of this compound, and Tomita ('32) in a later paper published similar results. Shipp and Zilva ('28), however, after a detailed investigation of the nitrogen intake and output concluded that, in the absence of vitamin C, there was no evidence of deranged nitrogen metabolism, and that any changes in nitrogen levels were due to the accompanying inanition.

It is obvious that the question of the effect of scurvy on nitrogen metabolism cannot be settled until there are con-current studies of the total nitrogen intake and the blood and urine nitrogen of scorbutic animals.

The literature contains very few references to analyses of the blood of scorbutic animals and the reason for this is attributed to the difficulties encountered in the collection of a sufficient number of blood samples during the experimental period. With the macro methods of analysis in common use such comparatively large quantities of blood are required that small animals would soon die of anemia. The intro-duction of micro methods, which can now be carried out with 0.1 and 0.2 cc. of blood, has made it possible to follow the course of diseases without undue depletion of the blood vol-ume. However, even when the micro methods are used with small quantities of blood the errors are so large, when colors are compared in an ordinary colorimeter, that it is impossible to interpret the results correctly.

In the present study, some of these sources of error have been minimized by using the Pulfrich photometer for colori-metric comparison. After the proper color filter for the particular determination has been decided upon and a con-centration curve has been prepared, successive determinations can be made with this instrument with remarkable accuracy and speed.

It was decided to confine this work, at first, to the deter-mination of those nitrogenous constituents of the blood which have previously been studied in the urine of scorbutic animals and reported by other workers, namely, uric acid, creatinine, urea, and also hemoglobin. The consensus of opinion, as

expressed by Myers, Fine and Lough ('16) is that the determination of the individual constituents of blood and urine gives much better insight into kidney function than does the measurement of the total non-protein nitrogen. This last determination when carried out photometrically with micro quantities, gives consistently high results, 30 to 38 mg. % of total non-protein nitrogen, in both normal and scorbutic blood. These results are not reported in detail at this time because the interpretation is uncertain. It should be explained, however, that in the procedure for the determination of non-protein nitrogen the sulfuric acid digestion and evaporation are followed by the use of Nessler's reagent. The resulting solution, although apparently clear in daylight, is distinctly turbid in the strong light of the photometer. It was thought that the high readings might be due to this slight turbidity, and not necessarily to high non-protein nitrogen content.

EXPERIMENTAL

Ten guinea pigs of fairly uniform size were selected and were adjusted to Eddy's ('29) modified Sherman, LaMer and Campbell basal ration. This diet was supplemented with green vegetables and tomato juice until it was determined that all of the animals were growing well. Six of the guinea pigs were then chosen for the experimental scurvy group and were fed the vitamin C free diet. Four others were fed the same diet and were given tomato juice, in addition, to provide the vitamin C. Unfortunately, two of the experimental animals were lost at an early date but the others followed the true scurvy course without complications. It was soon determined that the complete blood study could not be made during the short period of acute scurvy; consequently, the lives of the experimental animals were prolonged with doses of tomato juice at 4-day intervals. This lessened the precipitous growth decline and made possible a greater number of blood determinations. In this way the lethal stage was reached after a 6-week period rather than after 3 weeks. The growth curves of each animal showed a typical scurvy

picture and this was further substantiated by the gross lesions which were observed at autopsy. Figure 1, which follows, gives the individual growth curves of all the experimental animals. It is obvious that each of these follows the usual course.

The blood samples were taken from the ears of the guinea pigs, by capillary pipettes, after a small incision was made at the margin. The same ear was never used more than twice a week but several samples could be obtained from one in-
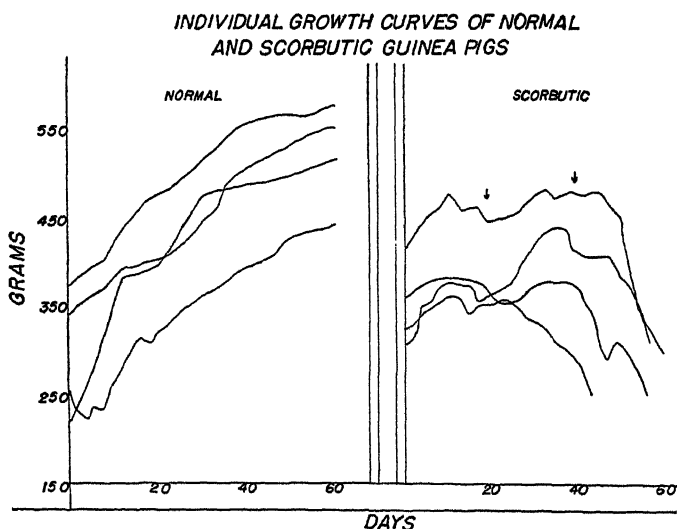
Fig. 1 The arrows on the scorbutic growth curves indicate the time when small doses of tomato juice were given to extend the scurvy period.

cision. Hemoglobin determinations were made at the same time, the blood being collected in a 5-cc. capillary diluting pipette. Throughout the work duplicate determinations were made frequently so that there could be no question as to the accuracy of the results. This was done both by the use of different portions of the same filtrate or, when necessary, with different blood samples taken at the same time. A total of 157 samples of blood was analyzed.

For each of the four constituents determined the procedure was as follows: a color absorption curve was first prepared

by determining the per cent of transmission with all of the nine color filters of the Pulfrich photometer. A filter showing suitable absorption was then selected, and a concentration curve was prepared by the use of standard solutions of three different concentrations. (In the case of hemoglobin a Newcomber plate was used instead of the standard solutions.)

TABLE 1

Blood determinations from animal no. 2—a normal subject

DATE	WEIGHT	HEMOGLOBIN	UREA NITROGEN	URIC ACID NITROGEN	CREATININE NITROGEN
	gm.	*%*	*mg. %*	*mg. %*	*mg. %*
3/ 5/36	302	93.0			
8	308				
11	326				
14	350				
17	360				
20	368		17.0	2.10	2.90
23	380				
26	391				
29	392				
4/ 1/36	401				
2	407				
4	...		15.0		3.0
5	...				
6	409				
7	...				
8	412				
9	...				
10	...	84.6	21.8		
11	416			2.50	
14	421		16.6		
16	426				
17	430				
4/18/36	439	83.9			

If the absorption of the colored solution follows the Lambert-Beers law, this curve should pass through the origin for zero concentration. With such a curve, the concentration of an unknown solution can be read directly, after the per cent of transmission is obtained in the photometer.

Urea. The standard calibration curve for urea nitrogen was made with different quantities of a standard ammonium

sulfate solution. The yellow color was produced according to Folin and Svedberg's ('30) method with Nessler's reagent. Color filter S 43 and the 10-mm. cup were used to make the transmission readings. The gum ghatti which is recommended

TABLE 2

Blood determinations from animal no. 6—scorbutic subject

DATE	WEIGHT	HEMOGLOBIN	UREA NITROGEN	URIC ACID NITROGEN	CREATININE NITROGEN
	gm.	%	mg. %	mg. %	mg. %
3/ 5/36	355				
8	365				
11	377	95.8			
14	401				
17	417	84.7			
20	437			2.30	
23	436			2.35	
26	409				
29	405				5.2
4/ 1/36	407	90.8	20.0	1.70	
2	...				
3	...		22.4	2.05	
4	407				
5	...			1.95	
6	394	89.7	18.0	1.65	
7	384		21.0		3.8
8	...				
9	...				
10	...				
11	356	88.0		1.40	3.6
12	...				
13	...				
14	334	66.0		1.95	
15	314			1.60	
16	306	71.9			3.0
17	300	75.9	19.0	2.30	
4/18/36	...			1.37	

in the Folin micro method was found not to be necessary for the comparative readings. If it is used, the colloidal appearance produced must be compensated by adding an equal quantity of the gum ghatti to the water solution which is placed in the comparison cup.

Uric acid. The calibration curve for uric acid nitrogen was obtained by making transmission measurements of different quantities of a standard uric acid solution with filter S 57 and a 10-mm. cup. The actual determination was performed as prescribed by Folin's ('33) newest micro method. Only 0.1 cc. of blood was used but the required depth of color was obtained by decreasing the dilution one-half.

Creatinine. The creatinine determination, at first, caused considerable trouble. The usual method of producing the red creatinine picrate by the use of alkaline picric acid required so much picric acid, that transmission readings could not be made in the photometer. The light was either entirely absorbed, or entirely transmitted. By reducing the concentration of the picric acid, it was found that filter S 50 would give transmission readings below 80% and above 5%. A corresponding dilution of the centrifuged filtrate was made by diluting 4 cc. of filtrate with 1 cc. of water. To this was added 3 cc. of an alkaline picrate solution (10 cc. 2% picric acid + 1 cc. 10% sodium hydroxide). The total volume was 8 cc. The correction for the absorption of the red isomer of creatinine picrate was made by using a standard solution of creatinine chloride treated with alkaline picric acid as described above and an alkaline picric acid blank in which water was used in place of the filtrate. Each had the total volume of 8 cc. The decreased transmission of the creatinine solution through color filter S 50, compared with the transmission of the picric acid blank was a measure of creatinine present. With larger concentrations of creatinine, longer standing was required to develop the full color, but the logarithmic curve was easily obtained.

Hemoglobin. The hemoglobin was determined with the aid of the standard Newcomber plate, but, instead of using this in the ordinary colorimeter, it was inserted in a frame and used as a standard for the photometric measurement. The calibration curve for the percentages of hemoglobin lay along a straight line between two limiting points on a logarithmic scale: the point for 100% hemoglobin, in which case the

standard and the sample gave identical readings at 100% transmission, and the point for 0% hemoglobin in which the transmission of the standard compared with water (0% hemoglobin) was only 51.7% for color filter S 50. With this modification, the photometric results proved much more accurate

TABLE 3

BLOOD DETER- MINATIONS, DATES	HEMOGLOBIN		UREA NITROGEN		URIC ACID NITROGEN		CREATININE NITROGEN	
	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
	%	%	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
3/ 8/36	93.0	89.0						
11	95.0	90.3						
14		88.7						
17		88.7	17.0			2.30		
20			15.0		2.10	2.21	2.90	
23					2.00	2.27	3.3	3.0
26					2.50			
29	94.0				1.63	1.87		
4/ 2/36		91.5		21.0		1.85		3.4
5	93.9	90.1	16.0	18.2		2.18	3.0	
8		87.0		16.8		1.78		2.9
11	84.6	87.3	18.9	16.6	2.30	1.58		3.3
14		78.1	16.6	19.0		2.06		3.2
17	89.0	73.9		17.8		2.07	2.85	2.9
Standard solution used in calibration	Newcomber glass standard		Ammonium sulfate, 1 cc. 0.01 mg. N		Folin's uric acid standard, 1 cc. 0.002 mg. uric acid		Creatinine chloride, 1 cc. 0.001 mg. creatinine	
Color filter used for the calibration	S 53		S 43		S 61		S.53	
Stratum thickness	10 mm.		10 mm.		10 mm.		10 mm.	

than identical measurements with the ordinary colorimeter when the same concentrations were used.

Tables 1 and 2 give a complete summary of the study made on a normal animal and on a scorbutic animal, respectively. These are typical of the results obtained for all the animals in each group.

Table 3 has been constructed to give a brief comparative picture of the two groups during the entire experimental

period. It gives average results of the determinations made on the animals of each group at the same stage in the experiment.

CONCLUSIONS

The work reported in this paper shows that the urea, uric acid, and creatinine in the blood of scorbutic guinea pigs did not rise above the normal level, and, therefore, no indication of abnormality in nitrogen metabolism was found in these animals. This substantiates the conclusions of Zilva (Shipp and Zilva, '28) and his co-workers, based on urinary studies, that there was no evidence of deranged nitrogen metabolism. On the other hand, these results are at variance with the theories of certain investigators whose work has been previously mentioned. Jarussowa ('26, '28), from her experiments with urine, thought that the nitrogenous waste products· were not excreted properly during the scorbutic period. Insufficient excretion implied accumulation of these products in the blood, but such increases have not been found. No evidence has been found, either, to support Nagayama's (Nagayama and Sato, '28) suggestion that the decrease in uric acid in the urine of scorbutic guinea pigs resulted from the accumulation of that waste product in the blood.

In the case of creatinine, the values found for both groups of animals are consistently somewhat higher than those usually reported as normal. This point is of interest in connection with the suggestion of Behre and Benedict ('35) that the chemical reaction involved in the creatinine-picrate method is not dependable.[1]

The results on hemoglobin are not in agreement with those of other workers, notably Mettier and Chew ('32), and Mouriquand, Leulier and Michel ('25) who have reported very serious anemias in scorbutic animals. The hemoglobin decreased, as was expected, but the anemia produced was never severe. This was interesting since the life of the animals was purposely prolonged in order to allow the slower development of scurvy and to give ample time for the study

[1] The new method proposed by these authors (Benedict and Behre, '36) was published after the completion of the present study.

of such accompanying pathological symptoms as anemia. It is believed that the hemoglobin technic employed in this work made it possible to detect any variations in hemoglobin more accurately than was possible by any methods which we have used heretofore.

Grateful acknowledgment is made to the department of zoölogy and physiology of Wellesley College for their coöperation in this work.

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THE GIZZARD FACTOR OF THE CHICK

H. J. ALMQUIST AND E. L. R. STOKSTAD

*Division of Poultry Husbandry, University of California College of
Agriculture, Berkeley*

It has been demonstrated (Dam, '35 a, b; Almquist and
Stokstad, '35 a, b; Almquist, '36; Dam and Schonheyder, '36)
that a hemorrhagic disease of chicks is due to the lack of a
new fat-soluble vitamin. In these investigations erosions of
the gizzard lining have been frequently found but their occur-
rence has not been closely correlated with the hemorrhagic
syndrome, since levels of anti-hemorrhagic vitamin supple-
ments which have sufficed to prevent hemorrhages and delayed
blood clotting have not prevented gizzard erosions. Gizzard
erosion has been commonly noted in the routine pathological
examination of chickens given the usual practical diets
(Jungherr, '35). Such erosions have been observed at this
laboratory in embryos in late incubation stages and in day-old
chicks (Holst and Halbrook, '33). On the other hand, the
hemorrhagic syndrome has not been reported in chicks reared
under practical conditions.

This gizzard disorder of chicks is characterized by erosion
or necrosis of the secreted gizzard lining, usually at the
cardiac end of the gizzard but often in other portions. The
erosions frequently are confined to the surface of the lining
but may be severe enough to penetrate the epithelium and the
muscular gizzard walls. The lining near the erosion is frayed
and loosened in most cases and may be brown or black colored.
The unaffected lining is usually quite coarsely ridged. In
view of the lack of evidence to classify these erosions as a

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distinct part of the hemorrhagic syndrome, or even to demonstrate clearly that they are the result of a dietary deficiency, further investigations have been made. A preliminary report of this work has already been given (Almquist and Stokstad, '36). Since the latter report, there has appeared a paper by Kline, Bird, Elvehjem and Hart ('36) on 'vitamin B_4' studies in which it is stated that dried hog lung tissue is an effective supplement for the prevention of gizzard lesions. Attempts to extract the factor were unsuccessful. These authors state that lack of the substance preventing occurrence of lesions in the gizzard has a profound effect upon the growth of chicks. They also advance the opinion that gizzard lesions may contribute to poor absorption.

METHODS AND RESULTS

The methods and procedure were the same as described in a previous paper (Dam and Schonheyder, '36). White Leghorn chicks were fed a basal diet composed of ether-extracted fish meal 17.5 parts, ether-extracted dried brewer's yeast 5.0 parts, ground polished rice 75.5 parts, cod liver oil 1 part and salt plus small amounts of ferrous and cupric sulphates 1 part. This diet was designated basal diet D. That this diet was adequate for normal growth was proved by the fact that the average weights of chicks at 4 weeks and at 6 weeks were frequently above the usual weight standards, even in lots deficient in the anti-hemorrhagic factor. The external appearance and development of all lots were likewise excellent. Mortality not associated with some phase of the hemorrhagic disease was a rarity. Chicks were reared for a standard period of 4 weeks, except for those chicks given the basal diet only, in which case the birds usually all died of the hemorrhagic disease in less than 4 weeks.

At the end of the period birds were killed and the gizzards removed and opened. An arbitrary standard for scoring the severity of the gizzard erosion was employed, a single plus sign representing a distinct erosion, two plus signs a severe erosion and three plus signs a very severe and wide-spread erosion. Doubtful cases were given a value of one-half plus.

The results of several preliminary experiments demonstrated conclusively that gizzard erosion was alleviated by high levels of a hexane extract of alfalfa and by whole alfalfa. A progressive decrease in severity of erosions was found as the dosage of the alfalfa hexane extract was increased by steps up to 25% equivalent, at which level complete or nearly complete prevention of erosions was obtained. Whole alfalfa at a 10% level did not prevent distinct erosions. At a 20% level of alfalfa, the erosions were much less severe but not completely prevented. The alfalfa hexane-extract was shown by separate assay with chicks to have an anti-hemorrhagic potency equivalent to that of the dried alfalfa from which it was prepared. The dosage of this extract required to prevent gizzard erosion was approximately 100 times the dosage required to prevent hemorrhages.

Certain other dietary supplements were tested for their influence both on hemorrhages and on gizzard erosions in an attempt to find a supplement which would prevent the latter without affecting the former. Results of these experiments are given in table 1.

From the data of table 1 it is evident that supplements rich in the known vitamins, fat and phospholipids failed to prevent gizzard erosions. Many of the supplements which prevented hemorrhages had no noticeable effect on gizzard erosions. On the other hand, the birds fed the fresh kale were entirely free from gizzard erosions. Dam ('35) has published data on a few birds fed orange meal which developed hemorrhages but no gizzard erosions. The above experiments with orange oil and with orange meal have differed from Dam's result.

A further series of experiments was conducted for the purpose of comparing the potencies of alfalfa, kale and hempseed extracts in preventing hemorrhage and gizzard erosion. The basal diet was slightly modified to the following composition: ether-extracted fish meal 17.5 parts, ether-extracted brewer's yeast 7.5 parts, ground polished rice 73.0 parts, salt mixture 1.0 part and cod liver oil 1.0 part, and was designated diet E. The procedure followed was the same as already described. The results of these experiments are given in table 2.

TABLE 1

Effect of certain dietary supplements on hemorrhages and gizzard erosions in chicks at 4 weeks of age

SUPPLEMENT TO DIET D	MANNER OF FEEDING	NUMBER OF CHICKS	EFFECT ON HEMORRHAGES	AVERAGE NUMBER OF + SIGNS PER GIZZARD
Cod liver oil	5% of diet	7	Negative	0.86[1]
Wheat germ oil, highly potent in vitamin E	5% of diet	10	Negative	1.40[1]
Orange oil	0.5% of diet	10	Negative	1.12[1]
Orange meal	5% of diet	10	Marginal	1.30[1]
Dried skim milk	5% of diet	10	Marginal	1.20
Chicken liver, vacuum dried, from chickens on normal diets	10% of diet	15	Marginal	0.80[1]
Liver extract[2]	100 cc. per kilogram of diet	8	Negative	1.10[1]
Fresh lemon juice	2 cc. orally per bird per day	11	Negative	0.96[1]
Egg yolk, hard boiled	One-fifth yolk per chick per day	15	Preventive	1.21
Egg white, hard boiled	One-fifth white per chick per day	15	Negative	1.10[1]
Fresh yellow carrots, no green portions used	ad. lib.	15	Negative	1.20[1]
Water extract of alfalfa	Equivalent to 10%	13	Negative	0.92[1]
Fresh kale	ad. lib.	11	Preventive	0.00
Hexane extract of alfalfa treated with magnesium oxide[3]	Equivalent to 40%	10	Preventive	1.10
Non-saponifiable alfalfa lipids	Equivalent to 40%	8	Preventive	1.28

[1] Several birds in this pen died with hemorrhages before 4 weeks. The gizzard scores for such birds have been included.

[2] Water extract of beef liver. One hundred cubic centimeters are equivalent to 1.7 kg. of whole liver. Kindly furnished by Dr. S. Lepkovsky.

[3] This treatment does not remove the anti-hemorrhagic vitamin.

Separate assays of the kale and alfalfa extracts showed that they were of nearly equal anti-hemorrhagic activity with the alfalfa extract being slightly more active than the kale extract. In comparison, hempseed extract contained little of the anti-hemorrhagic vitamin since it did not impart complete protection at a 5% equivalent level.

TABLE 2

Comparative potencies of alfalfa, kale and hempseed hexane extracts in preventing hemorrhages and gizzard erosions

SUPPLEMENT TO BASAL DIET E	EQUIVALENT PER CENT	NUMBER OF CHICKS	AVERAGE CHICK WEIGHT IN GRAMS	INCIDENCE OF HEMORRHAGES, PER CENT OF BIRDS	AVERAGE NUMBER OF + SIGNS PER GIZZARD	TOTAL MORTALITY, PER CENT OF BIRDS
None	..	8	....	100.0	0.94	100.0[1]
Alfalfa extract	¼	8	198.8	0.0	1.10	0.0
Alfalfa extract	½	8	186.2	0.0	0.85	0.0
Alfalfa extract	20	8	204.4	0.0	0.29	0.0
Alfalfa extract	25	8	197.5	0.0	0.13	0.0
Kale extract[2]	¼	8	196.5	0.0	0.44	0.0
Kale extract	½	8	201.3	0.0	0.31	0.0
Kale extract	5	8	190.0	0.0	0.19	0.0
Kale extract	15	8	201.3	0.0	0.06	0.0
Kale extract	25	8	221.2	0.0	0.06	0.0
Hempseed extract[4]	¼	8	203.4	62.5	1.10[3]	62.5
Hempseed extract	½	8	205.0	75.0	1.40[3]	50.0
Hempseed extract	5	8	197.3	12.5	1.00[3]	12.5
Hempseed extract	15	8	215.2	0.0	0.44[3]	12.5
Hempseed extract	25	8	210.0	0.0	0.25	0.0

[1] All birds died with hemorrhagic symptoms in less than 14 days.
[2] Prepared by thorough hexane extraction of vacuum-dried kale. Equivalent per cent refers to dried kale.
[3] Gizzard scores for birds which died have been included.
[4] Prepared by thorough hexane extraction of ground hempseed. Equivalent per cent refers to whole hempseed.

From the gizzard erosion standpoint, the kale extracts appeared relatively more active than the alfalfa extracts. The kale extract at a 5% equivalent level was about as effective in preventing erosion as the alfalfa extract at 25%. At lower dosages, the kale extract was consistently more effective on erosions than the alfalfa extract. Further trials at 5% equivalent levels of each extract gave similar results. The mortality

on the lower levels of hempseed extract was so severe that comparisons cannot safely be made. There was a decided reduction in severity of gizzard erosion at the higher levels of hempseed extract.

The amount of alfalfa extract required to prevent erosions as compared to that required to prevent hemorrhages was in a ratio of at least 100 to 1, respectively, while the corresponding ratio for the kale extract was approximately 20 to 1. The results obtained with the kale and alfalfa extracts, in particular, indicated that the fat-soluble factor influencing gizzard erosion was not the anti-hemorrhagic vitamin since these extracts, although equal in anti-hemorrhagic activity, were of distinctly different potency in preventing gizzard erosion. The fact that the factor which alleviated gizzard erosion was not in the non-saponifiable fraction or in the filtrate from the activated magnesium oxide treatment (table 1) constituted a further proof of its difference from the anti-hemorrhagic vitamin.

Experiments were next conducted to determine if this factor could be detected in the saponifiable lipid fraction. For this purpose chicks were kept on the basal diet for 1 week, then two groups of chicks were given preparations of kale lipids, one the non-saponifiable fraction equivalent to 20% dried kale and one the saponifiable fraction at the same dosage. A third group was given 1% equivalent of alfalfa hexane extract. After 2 weeks of such feeding, the birds were killed and the gizzards examined as before. The group receiving the non-saponifiable kale lipids had an erosion score of 1.10, that receiving the saponifiable kale lipids a score of 0.19, and that receiving the alfalfa extract 1.13. The gizzard score of 0.19 in the group fed the saponifiable fraction was due to three doubtful cases which probably were in the process of healing. This experiment showed that the anti-gizzard-erosion factor was not in the non-saponifiable fraction and was definitely present in the saponifiable fraction.

It was also found that, in contrast to the anti-hemorrhagic vitamin (Dam, '35 a, b; Almquist and Stokstad, '35; Almquist,

'36), the gizzard factor was destroyed readily by heat. Preparations of dried kale which were heated for 24 hours in a vacuum dryer at more than 100°C. completely lost their power to prevent gizzard erosion. The anti-gizzard-erosion potency of the hexane extracts was also adversely affected by saponification and only the most careful treatment during saponification would preserve some of the potency.

TABLE 3

Effect of certain supplements on gizzard erosions in the presence of the anti-hemorrhagic vitamin

SUPPLEMENT TO DIET E PLUS THE ANTI-HEMORRHAGIC VITAMIN	MANNER USED	NUMBER OF CHICKS	AVERAGE NUMBER OF + SIGNS PER CHICK
Wesson oil	5% of diet	16	0.85
Corn oil	10% of diet	8	1.00
Linseed oil	5% of diet	10	0.65
Hempseed oil	5% of diet	10	0.65
Walnut oil	5% of diet	10	0.60
Lard	5% of diet	10	0.85
None		10	0.95
Hog liver, air dried, 35°C.	5% of diet	9	0.72
Hog lung, air dried, 35°C.	7% of diet	10	0.85
Hog kidney, air dried, 35°C.	3% of diet	11	0.82
Hog heart, air dried, 35°C.	4% of diet	10	0.85
Hog pancreas, air dried, 35°C.	1.5% of diet	10	0.80
None		10	0.88
Hog lung, vacuum dried	5% of diet	10	1.06
None		10	1.11
Alfalfa ash	Equivalent to 25%	9	0.91
Cotton pulp	5% of diet	9	1.13
Calcium carbonate	5% of diet	9	0.94
Dried skim milk	5% of diet	15	0.93
Dried cabbage	5% of diet	9	0.90
None		12	1.02

The next experiments were designed principally to test for other sources of the gizzard factor using diet E supplemented with a plentiful amount of the anti-hemorrhagic vitamin. This was supplied in the form of kale or alfalfa hexane-extract treated with activated magnesium oxide and at a level equivalent to 2% of the dried leaf tissue. Results of these experiments are given in table 3.

The information summarized in table 3 indicates some slight preventive action in several of the oils but not sufficient to establish any of these oils as a practical supplement. The various air-dried hog tissues gave results not appreciably different from the control group. The vacuum-dried hog lung was also ineffective although prepared in a manner similar

TABLE 4

Effect of wheat bran, wheat bran hexane extract and alfalfa hexane extract on gizzard erosions

	GROUP			
	A	B	C	D
Diets				
Fish meal	17.5	17.5	17.5	17.5
Ground polished rice	58.0	73.0	58.0	58.0
Brewer's yeast	7.5	7.5	7.5	7.5
Wheat bran	15.0	...	...	15.0
Hexane extract of wheat bran, equivalent level	...	30.0	...	...
Wheat bran, hexane extracted	...	...	15.0	...
Salt mixture	1.0	1.0	1.0	1.0
Cod liver oil	1.0	1.0	1.0	1.0
Wesson oil	2.0	2.0	2.0	2.0
Alfalfa hexane extract treated with activated magnesium oxide, equivalent level	2.0	2.0	2.0	...
Alfalfa hexane extract, equivalent level	...	...	...	20.0
Number of chicks	9	9	9	10
Mortality	0	0	0	0
Average weight of chicks at 40 days in grams	318.0	317.5	334.0	319.2
Average number of + signs per gizzard	0.67	0.25	1.25	0.10
Texture of gizzards	Fine	Coarse	Fine	Fine
Average weights of gizzards in grams	8.9	7.5	9.3	8.6

to that of Kline, Bird, Elvehjem and Hart ('36). Growth was practically equivalent in all comparable lots.

Experience with practical chick diets led to the observation that diets containing wheat bran in addition to the dried alfalfa were productive of very slight erosions, if any. We accordingly investigated wheat bran. Results of an experiment in this connection are given in detail in table 4.

In this experiment chicks were raised to 12 days of age on diet E plus an adequate supply of the anti-hemorrhagic vitamin. A sample of these 12-day-old chicks was examined for gizzard erosion and found to have an average score of 1.06 (fifteen birds). Four groups of the most vigorous remaining chicks were selected and placed on diets as listed in table 4. They were kept on these diets for 4 weeks.

Inspection of these data shows that growth was equally good in all lots irrespective of the condition of the gizzards. Similar results are shown in table 2. Group A fed the whole wheat bran alone had definitely better gizzards than group C fed the extracted wheat bran. Group C had the most severe erosions, representing a complete lack of curative ability in the diet. The results with group B showed that the curative effect of wheat bran was localized in the hexane-soluble fraction. When whole bran was reinforced by a hexane extract of alfalfa, as in group D, nearly perfect gizzards resulted. The small scores in groups B and D are due to a few cases in which healing was not complete but formation of new lining at the location of the erosion was apparent.

The gizzards obtained from groups A, C and D were all well packed with fiber while those of group B were soft and flabby. The erosion score showed no relation to the presence of fiber or bulk in the diet, in agreement with results we have obtained with cotton pulp (table 3). Groups A, C and D exhibited a finely ridged texture and group B a coarsely ridged texture. Group B had the smallest gizzards. These characteristics also appeared unrelated to the erosions. We have made other observations which confirm these findings. Representative gizzards from these groups are illustrated in figure 1.
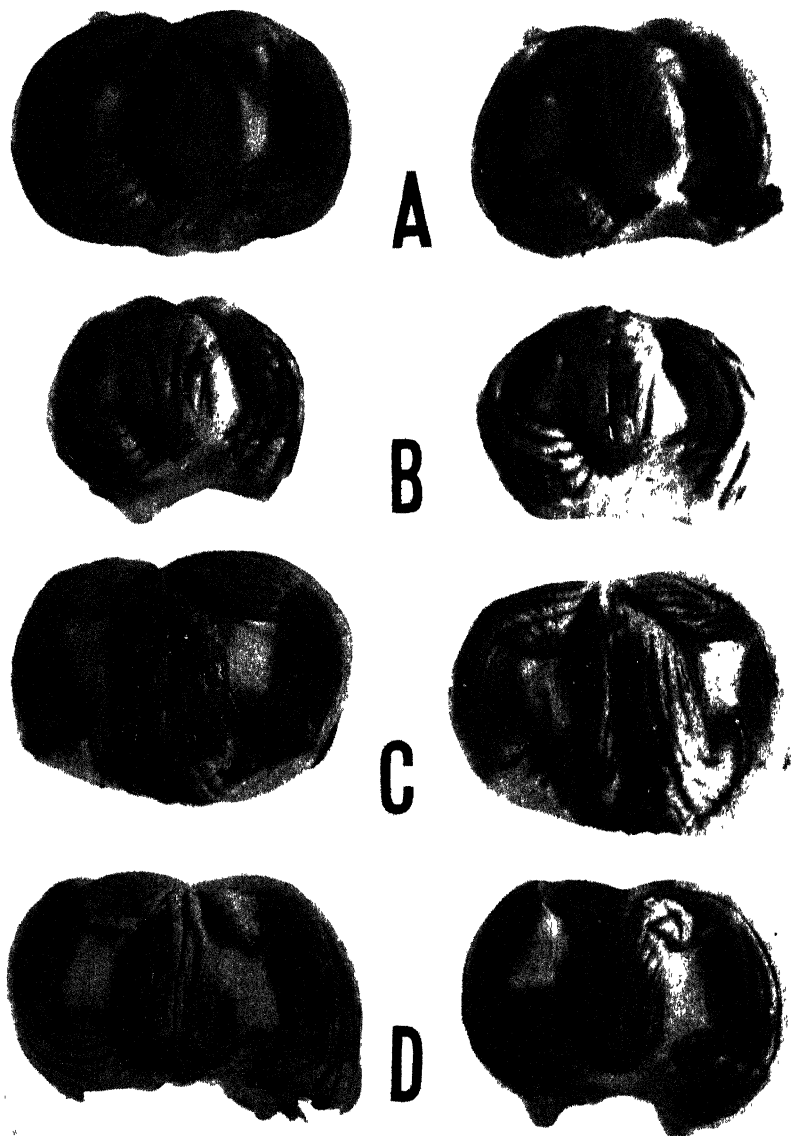
Fig. 1   Interior views of representative gizzards from group A—whole wheat bran, group B—wheat bran hexane extract, group C—hexane-extracted wheat bran, and group D—whole wheat bran and alfalfa hexane extract.

DISCUSSION

It has been shown in the preceding section that a comparatively unstable, fat-soluble factor is required by chicks for proper formation of the gizzard lining. Absence of this factor in the diet leads to erosions or lesions of the lining. Lesions can be prevented or cured by providing an adequate level of the gizzard factor. The factor can be clearly differentiated from vitamins A, D, E, F, the anti-hemorrhagic vitamin and the anti-encephalomalacic vitamin of Goettsch and Pappenheimer ('36). In comparison to the latter, the gizzard factor differs distinctly in occurrence, properties and pathology. At the present time, fresh or dried greens and wheat bran seem to be the best practical sources.

All of our data and observations lead to the conclusion that the gizzard factor is not a growth factor. Jukes ('37) has similarly observed that gizzard lesions do not affect growth. We are unable to explain the better growth observed by Kline, Bird, Elvehjem and Hart ('36) in birds given dried hog lung tissue as a preventive for gizzard lesions. It is possible that these workers may have unwittingly rectified their diets in respect to some other factor by the use of dried hog lung. Contrary to their report, we find no anti-gizzard-erosion activity in this supplement.

SUMMARY

1. Gizzard erosions or lesions of the chick are caused by a deficiency in the diet of a fat-soluble factor. This factor is not identical with any of the known vitamins.

2. The gizzard factor appears to be comparatively unstable being easily destroyed by heat and by alcoholic potash. It is readily adsorbed from solution in hexane by activated magnesium oxide. It appears to be in the saponifiable fraction.

3. The best practical sources of this factor yet found are fresh and dried greens and wheat bran.

4. Presence or absence of gizzard erosions or of the gizzard factor in the diet has no appreciable effect on the growth of chicks.

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THE EFFECT OF LOW FAT DIETS ON SERUM LIPIDS OF RATS [1]

ARILD E. HANSEN AND WILLIAM R. BROWN [2]

Departments of Pediatrics and Botany, University of Minnesota, Minneapolis

Although it is well known that the character of the body fats may be changed by dietary measures, very little definite information is available to indicate that the blood lipids may be similarly affected. Hansen and Burr ('33) observed that the serum lipids of rats fed on fat-free diets were of a lower degree of unsaturation than those of control animals on stock diet. Similar results were obtained by Williams and Maynard ('34) in their lactation studies on goats. Recent investigations by the authors indicate that the same phenomenon occurs in the human subject. The present report concerns a more detailed study of the relation between the diet and the serum lipids.

Female animals of the Wistar strain were used. The conditions as to environment and routine were the same as described previously (Burr and Burr, '29). Control rats were reared on the stock diet of McCollum and Simmonds ('18). This diet contains approximately 4.5% fat, of which the total extractable fat has an average iodine value of 58. In one group of animals on this diet, the food intake was so restricted as to keep their weight at the same level as that of the average of the fat-free rats of the same age. A second group of animals on the same diet was sacrificed at 2 months of age. The 550 B diet of Burr and Burr ('29) with a total fat content of less than 0.01% was used for the fat-free regimen. The ani-

mals on this diet showed the typical findings of the fat deficiency syndrome. The esters of oleic acid and linolic acid were administered in various small quantities to several groups of animals on the fat-free diet.

Uniform fasting periods of 8 hours were used throughout the study. After mild chloroform anaesthesia, a small window

TABLE 1

Serum lipids of rats fed on stock diet

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
A. Normal adult rats					
	mg./100 cc.	mg.%	mg.%	mg.%	
147	325	108	193	301	108
10	431	111	280	391	111
15	428	116	271	387	111
470	548	84	306	390	140
13	308	74	209	283	109
246	554	113	367	480	115
219	384	74	246	320	117
338	446	60	275	335	133
472	409	74	287	360	114
11	277	68	174	242	114
12	320	71	200	271	118
41	478	118	292	410	117
39	438	102	256	358	123
23	410	80	265	345	119
14	482	79	321	401	120
Average	416	88.8	263	352	118
B. Normal rats 2 months of age					
1	393	79	309	388	101
2	415	107	267	374	111
3	421	99	278	377	112
24, 25	422	103	265	368	115
Average	413	97	280	388	110
C. Adult rats held to weight level of average of fat-free animals					
73	422	98	199	297	142
47	435	85	238	343	127
84	412	91	220	311	132
740	439	99	221	320	137
Average	427	93	220	318	134.5

was cut in the ventral thoracic wall and the heart was made to herniate. The heart was incised and the blood was collected directly into a test tube, gentle pressure being applied to the body to insure maximum collection of blood. For the most part the individual specimens were kept separate, however, at times large samples were obtained by pooling the blood from several animals of the same group. The blood

TABLE 2

Serum lipids of rats fed on the fat deficient diet

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
	mg./100 cc.	mg.%	mg.%	mg.%	
330	262	84	212	287	91
308	330	75	252	337	98
55	269	87	285	372	72
73	267	78	239	317	84
77	267	86	232	318	84
63	431	129	360	489	88
74	329	81	282	363	91
75	374	84	285	443	84
67	343	92	335	377	91
155	423	140	283	423	100
136	330	100	218	318	103
65	282	93	200	293	96
53	393	110	290	400	98
56	308	98	211	309	100
59	328	68	231	299	109
55	269	87	285	372	72
73	267	78	239	317	84
77	267	86	232	318	84
60	530	106	437	543	97
Pooled (4)	305	84	274	358	85
Pooled (5)	310	66	300	366	85
Average	327.8	91.0	270.6	362.3	90.3

was allowed to clot, and the serum obtained by centrifuging for 20 minutes at 800 r.p.m. The alcohol-ether (3 : 1) extract was prepared immediately after the manner of Bloor. The cholesterol was determined on aliquots of the alcohol-ether extract using the Lieberman-Burchart reaction according to the method described by Bloor ('28). Aliquots of the original extract were saponified, acidified and extracted with petroleum

ether and the total fatty acids determined by the oxidative method of Bloor ('28). The iodine absorption value of the serum was obtained on aliquots of the alcohol-ether extract using the Rosenmund-Kuhnhenn ('23) method as modified by Page, Pasternak and Burt ('30). Yasuda's technic was followed except that N/100 instead of N/20 sodium thiosulphate

TABLE 3

Serum lipids of rats fed on the fat deficient diet plus esters of oleic and linolic acid

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
A. Methyl oleate 13 drops daily					
	mg./100 cc.	mg.%	mg.%	mg.%	
110	394	82	295	347	113
115	464	113	411	524	89
116	637	130	432	562	113
109	461	98	344	442	104
111	438	98	302	400	109
Average	478.8	104.2	356.8	455.0	105.6
B. Methyl linolate ½ drop daily					
137	478	105	370	475	100
144	501	73	456	529	95
149	408	111	423	535	76
Average	462.3	96.3	416.3	513	·90.3
C. Methyl linolate 1 drop daily					
150	314	75	263	338	93
122	311	83	252	334	93
142	552	85	429	514	107
132	369	87	300	387	95
Average	386.5	82.5	311.0	393.25	97.0

was used for the titration. The total lipids represent the sum of the cholesterol and total fatty acids. The iodine number of the total lipids was obtained by dividing the milligrams iodine absorbed per unit of serum by the milligrams of total lipid per unit. Duplicate determinations were made on the specimens from the larger rats and on the pooled samples. The results of the experiment are presented in tables 1 to 3.

DISCUSSION

The values for the cholesterol and total fatty acids were essentially the same, both in the rats which had been fed on the highly purified, fat-free diet and in the control animals reared on the stock diet. The total iodine absorbed per unit of serum, however, was lower in the animals of the fat-free group. The iodine number of the total lipids was definitely lower in the group which had been reared on the fat deficient diet (tables 1 and 2). In that cholesterol has an iodine value of 66, it is possible to calculate the iodine value of the total fatty acids of the serum. By this means the average iodine number of the serum fatty acids of the fat-free animals was found to be 103 as compared with 135 for control animals on stock diet. In the larger samples of blood obtained by pooling the serum of several animals, it was found that the iodine number of the total lipids for the fat-free animals was definitely low (table 2). The iodine number of the total fatty acids tends to be lower in the younger age group in human beings (Hansen, '37). A similar finding was obtained in the group of four animals 2 months of age (table 1). The iodine numbers of the total lipids were definitely higher in the group of animals (table 1) which had been given the stock diet only in quantities sufficient to allow them to grow at a rate comparable to the average of that of the fat-free group. The serum cholesterol values in this group were found to be unchanged but the total fatty acids were definitely low. The authors in studying the effect of a high-fat diet in rats observed that the level of the fatty acids of serum tends to vary inversely with their degree of unsaturation. The significance of this is not definite since the character of the body lipids has not as yet been determined. However, it suggests the possibility that the more saturated fats have been utilized during the period of partial starvation.

Esters of linolic acid produce a striking improvement in the condition of the rats suffering from the unsaturated fatty acid deficiency disease. Even when given in small amounts, the animals resume their normal rate of growth, the hematuria disappears and the skin condition clears up. A group of

animals was placed upon various small quantities of the methyl esters of linolic acid and the serum lipids were determined. The findings of this study are presented in table 3. The group of animals which had been given 1 drop daily of the linolic acid ester continuously with their fat-free diet were healthy appearing animals, but the average iodine number of blood lipids was only slightly raised. The rats which had been given only ½ drop of methyl linolate were found to have a symptomatic cure, but the blood lipid iodine numbers were essentially the same as those of the fat-free group. These observations indicate that when the esters of linolic acid are given in minute quantities to rats on the fat-free ration, although they produce a symptomatic cure, they cause only a slight increase in the iodine value of the serum lipids.

The esters of oleic acid were fed in larger amounts than were the esters of linolic acid. There was a slight clinical improvement in these rats although they were by no means normal, healthy animals. Esters of oleic acid are difficult to prepare in pure form, linolic acid esters being the most likely contaminant. Traces of linolic esters present in the methyl oleate preparations may have been responsible for the improvement that occurred in these animals (Burr, Burr and Miller, '32). The iodine values of the serum lipids were somewhat higher than those of the animals given the linolic ester supplements (table 3). The fact that the esters of oleic acid were administered in larger amounts than were the esters of linolic acid no doubt accounts for the greater degree of unsaturation of the serum lipids in this group.

SUMMARY AND CONCLUSIONS

1. The serum lipids of rats reared on a fat-free diet have a lower degree of unsaturation than do the serum lipids of rats on stock diet.

2. Young animals tend to have a lower iodine number of the serum lipids than do normal adult animals on the same diet.

3. When the food intake of the animals on stock diet was so restricted as to hold their weight to the level of the average of the fat-free group, it was found that the degree of unsaturation of the total lipids of the serum was greater than in the normal animals on the same diet unrestricted.

4. Animals given small quantities of the methyl esters of linolic acid sufficient to effect a clinical cure of the unsaturated fatty acid deficiency disease were found to have a slight but definite increase in the iodine number of the total lipids.

5. Esters of oleic acid given in fairly large quantities to animals on the fat deficient diet were able to cause a definite increase in the iodine values of the total lipids, even though they effected only a partial clinical cure.

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REVIEW

RECENT STUDIES OF VITAMINS REQUIRED BY CHICKS *

THOMAS H. JUKES

Division of Poultry Husbandry, University of California, Davis

The nutritional requirements of chicks have long been known to be incompletely supplied by purified diets which are suitable for rats. Recent investigations indicate that some of the missing factors are being found. For example, within the past 2 years the antihemorrhagic vitamin, essential for the chick, but not found necessary in rat diets, has been purified to a high degree. Comparative studies with rats and chicks have paved the way for the discovery of the different forms of vitamin D. Such comparative studies promise to be of assistance in unravelling the complexities of the water-soluble group of vitamins.

Many papers which deal with poultry nutrition are published annually, and it is beyond the scope of this review to deal with more than a small proportion of such reports. An attempt has been made to confine the review a) to investigations dealing with new dietary essentials, usually of a vitamin-like nature, and b) to recent reports of quantitative experiments with the older and better-known vitamins.

* Exigencies of space have made it necessary to shorten the Literature Cited list of this review. The references indicated by superior numerals may be found in the bibliographies of the following articles:

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1. The antihemorrhagic vitamin ('vitamin K')

A tendency toward delayed blood clotting in chicks fed on certain simplified diets was observed by Dam ('29) and by McFarlane and co-workers ('31). The latter investigators noted that the condition was characteristic of the feeding of protein supplements which had been extracted with ether. Holst and Halbrook ('33)[6] also described the condition, and found that a cure was brought about by feeding small quantities of cabbage. Dam and Schonheyder ('34)[4] produced the dietary hemorrhagic disease in chicks, and showed that the disease was not caused by a deficiency of any of the known vitamins. Dam ('35) and Almquist and Stokstad ('35),[2] working independently, noted that the protective factor was fat-soluble and unsaponifiable. Dam found the factor to be present in hog liver fat, hempseed, tomatoes and kale. He suggested that it be named 'vitamin K' (Koagulations vitamin). Almquist and Stokstad found that an ether extract of dehydrated alfalfa was an excellent source of the factor, and that the factor was synthesized in fish meal when it was allowed to putrefy slightly, potent extracts of such fish meal being obtained. The vitamin did not promote growth. Later work by Almquist ('36 a) reported concentration of the vitamin from a hexane extract of alfalfa meal. A reddish oil was obtained which was treated with digitonin to remove a small quantity of sterols. The oil was bleached to a light yellow color by treatment with activated magnesium oxide. It afforded complete protection against hemorrhages when fed at a level of 2 parts per million of diet. The factor appeared to be stable to heat and light.

Dam and Schonheyder ('36) also prepared concentrates of the vitamin from extracts of dried alfalfa. They removed inactive material from a light petroleum solution by shaking with 90% methanol. The vitamin was then adsorbed on sugar, which brought about a fivefold concentration. They confirmed Almquist's ('36 a) report that the vitamin was rapidly destroyed by alcoholic potash.

Schonheyder ('36) proposes for vitamin K a unit which is, in substance, that amount of the vitamin which is required per gram of a deficient chick to be orally administered on 3 successive days in order to render normal the clotting power of the blood. A deficient chick weighing 333 gm. would therefore require 1000 units. Almquist's ('36 a) quantitative estimations are based on the level which must be fed in the basal diet to protect chicks against hemorrhages. It is difficult to compare the potency of Almquist's concentrate with Dam and Schonheyder's material, but, on the basis of feed intake, it appears that Almquist's concentrate has a potency of about 10^7 Schonheyder units per gram, and is therefore the most potent concentrate yet reported. More recently, Almquist ('36 b) has reported a several-fold increase in potency produced by distillation of his concentrate in a molecular still.

Some physical and chemical properties of highly potent concentrates of the vitamin, purified by distillation in vacuo were described by Almquist ('37). Molecular weight determination by the camphor method gave a value of about 600. The concentrate contained a trace of nitrogen; phosphorus and sulphur were absent. The light yellow color of the concentrate was attributed to carotene, the presence of which was demonstrated. Other evidence indicated that the antihemorrhagic vitamin is colorless. It was not affected by treatment with phenyl isocyanate or di-nitro benzoyl chloride, reagents for the hydroxyl group, or by di-nitro phenyl hydrazine. Its potency was destroyed by cold bromination and by perbenzoic acid, indicating the presence of unsaturated linkages, and by direct sunlight. The vitamin appeared to be a very complex substance primarily hydrocarbon in structure.

Almquist and Stokstad ('36 b) described conditions influencing the incidence of the hemorrhagic syndrome. The vitamin was found in chick droppings collected in a bactericidal solution, although the chicks were on a diet deficient in the vitamin. This showed bacterial synthesis of the factor in the intestine. Transfer of the vitamin to the chick from the diet of the hen was shown to take place via the egg yolk. The livers

of young chicks on normal diets contained no appreciable quantity of the antihemorrhagic vitamin.

Mode of action of the antihemorrhagic vitamin. The current explanations of normal blood clotting as given in an ordinary physiological textbook consist merely of applying names and ascribing functions to certain crude blood and tissue fractions. Consequently the mode of action of 'vitamin K' is an unsolved physiological problem of the greatest interest. Schonheyder ('36) and Dam, Schonheyder and Tage-Hansen ('36) have studied the question, and reported that the prothrombin fraction from normal chick plasma brought about clotting of deficient plasma in vitro. Concentrates of 'vitamin K' itself were not active in vitro, but the vitamin was present in the prothrombin fraction of normal plasma. It could not be removed from this fraction by washing with acetone and ether, although the vitamin in the free state is soluble in these solvents.

2. Gizzard erosions of dietary origin in chicks

It was reported by McFarlane and co-workers ('31)[2] that the lining membranes of the gizzards of chicks fed on certain simplified diets were eroded and exfoliated. Gizzard erosions in chicks were also reported by Ringrose, Norris and Heuser ('31),[1] in their description of a pellagra-like syndrome in chicks, and by Holst and Halbrook ('33),[6] Dam and Schonheyder ('34)[4] and Almquist and Stokstad ('35)[2] in their respective accounts of the hemorrhagic syndrome. When the basal diets described in these five reports are compared, it is interesting to note that all of them were free from leafy materials, such as alfalfa meal, which are commonly found in practical chick rations. However, Jungherr ('36) reported the condition in chickens given the usual practical rations.

Steps toward the preparation of a dietary factor preventing gizzard erosions were described by Almquist and Stokstad ('36 a).[2] The saponifiable fraction of kale lipids was found to be a potent source of the factor, while copious amounts of the known vitamins, including the antihemorrhagic vitamin,

were without effect. Kline and co-workers ('36),[4] working with an entirely different basal diet, reported that dried lung tissue protected against gizzard erosions, and that the potency was not extracted from lung tissue with hot water. They state that lack of the substance preventing occurrence of lesions in the gizzard had a profound effect upon growth of chicks, due to 'poor absorption.' Almquist and Stokstad ('35),[2] however, found gizzard erosions to occur in rapidly growing birds, and Jukes ('36)[2] stated that growth was not affected by gizzard erosions. The fact that the gizzard is not essential to the absorption of food has been clearly shown by Fritz, Burrows and Titus ('36), who found excellent health to persist in gizzardectomized birds.

Almquist and Stokstad ('37), in a recent publication, report that the factor preventing gizzard erosions is fat soluble. They found it to be easily destroyed by heat and to some extent by alcoholic potassium hydroxide. It was readily adsorbed from solution in hexane by activated magnesium oxide. The best practical sources of the factor were fresh or dried green leaves, and wheat bran. Chick diets containing adequate amounts of wheat bran and dried alfalfa produced very slight, if any, gizzard erosions.

In a recent article, Bird, Kline and co-workers ('36), state that the anti-gizzard-erosion factor was insoluble in ether and in ethyl alcohol, but followed the alkali-soluble acid-precipitable proteins in the fractionation of lung tissue, which was one of the best sources of the factor. Pork liver and kidney were also excellent sources, and oats, wheat bran and middlings were superior to wheat and corn. The factor was quite thermostable in lung tissue, but was destroyed readily in grains either by dry heat or autoclaving. From these results one is led to the conclusion that the factor described by the Wisconsin group must be different from the factor of Almquist and Stokstad ('36 a),[2] but comparison of results on widely differing basal rations is extremely difficult.

3. Nutritional encephalomalacia

It was observed by Drummond ('16)[6] that chicks could not live on a 'simplified diet.' Many unsuccessful attempts have since been made to devise 'purified diets' for chicks. Failure usually results when a purer source of carbohydrate such as starch, dextrin or sucrose is used in place of grain in a chick diet. Recently Hogan, Boucher and Kempster ('35) have been able to raise chicks on 'simplified' rations, which contained such materials as casein, cornstarch, cellophane, lard, wheat germ oil, acid hydrolyzed yeast, liver extract, tikitiki, salt mixture, carotene and irradiated ergosterol.

One of the earlier complications usually observed in chicks on purified diets is characterized by acute paralytic symptoms which simulate polyneuritis. The symptoms and pathology of this nutritional disorder ('encephalomalacia') which is quite distinct from vitamin B deficiency, were first described by Pappenheimer and Goettsch ('31).[6] The most characteristic macroscopic post-mortem symptom is the existence of discolored spots on the surface of the cerebellum, and edema of the brain.

The discovery of a preventive factor in vegetable oils was announced by Pappenheimer and Goettsch ('34). A simultaneous report by Adamstone ('34) stated that the symptoms could be produced by feeding a diet which had been subjected to the Waddell and Steenbock ('28) treatment with ferric chloride for the destruction of vitamin E. Goettsch and Pappenheimer ('36) stated that the non-saponifiable fraction of soy bean oil was a good source of the anti-encephalomalacic factor and that fractions could be prepared which would protect when fed at a level of 0.024%. The factor was stable to autoclaving. Saponification of soy bean oil destroyed the factor unless air was excluded. In the original vegetable oil, however, it was resistant to aeration. Ninety-five per cent alcohol extracted much of the factor from vegetable oil. Corn, cotton seed, peanut and soy bean oils all contained the factor. In contrast to the findings of Adamstone ('34), nutritional encephalomalacia was not produced in chicks by feeding a natural diet

in which vitamin E had been completely destroyed by ferric chloride treatment. Addition of certain foodstuffs rich in vitamin E did not protect chicks on the basal simplified diet, which consisted of starch, dried skim milk, commercial casein, lard, bakers' yeast, paper pulp, salt mixture and cod liver oil.

Vitamin B_4. A water-soluble factor, designated 'vitamin B_4,' was stated by Keenan, Kline, Elvehjem, Hart and Halpin ('33)[4] to be necessary to prevent paralysis in chicks. The paralysis developed on a diet of casein, dextrin, salt mixture, yeast and cod liver oil, and was accompanied by a degeneration of the brain similar to that described by Pappenheimer and Goettsch ('31).[6] A later communication by Kline, Bird, Elvehjem and Hart ('36)[4] describes an improved basal ration for the study of nutritional paralysis in chicks consisting of dextrin, purified casein, salt mixture, brewers' yeast, autoclaved liver residue, crude liver extract powder, water-extracted lung tissue and cod liver oil. The addition of 15% of peanuts, brain or kidney produced normal chicks and fair success was reported with 'certain B_4 concentrates' (undescribed). It is not yet possible to suggest any relationship between the syndrome described by Pappenheimer and Goettsch and that observed by the Wisconsin workers, and characterized by similar symptoms. Goettsch and Pappenheimer ('36) point out that the fact that their preventive factor was present in the non-saponifiable fraction of soy bean oil excludes the possibility that it was 'vitamin B_4' or any other water-soluble vitamin.

Once again, the differences in the basal diets used by different laboratories make comparison of the results difficult. If the two factors are different, it should be possible, to devise a diet which is deficient in both of them, and which will permit normal development only when supplemented with both the vitamin of Pappenheimer and Goettsch and with the 'vitamin B_4' of the Wisconsin workers. In the meantime, it is worthy of note that Kline, Bird, Elvehjem and Hart ('36) reported that hydrogenated cottonseed oil[7] was protective against 'vitamin B_4' deficiency and Arnold, Kline,

[7] Crisco.

Elvehjem and Hart ('36) fed peanut oil 'to supply vitamin B_4.' Goettsch and Pappenheimer ('36) had previously observed that these oils contained their fat-soluble anti-encephalomalacic factor.

4. Vitamin G and associated factors

The need of the chick for a thermo-stable member of the vitamin B complex was first demonstrated by Hauge and Carrick ('25).[1] A pellagra-like syndrome in chicks was produced by Ringrose, Norris and Heuser ('31),[1] who showed that the symptoms could be prevented by including autoclaved yeast or a 'milk vitamin concentrate' in the diet. Later work by Norris and associates (Norris, Heuser, Ringrose, Wilgus and Heiman, '34;[1] Wilgus, Norris and Heuser, '35 a;[1] Davis and Norris, '36) and by Bethke and co-workers (Bethke, Record and Kennard, '33, '36;[1] Bethke, Record and Wilder, '36 a) demonstrated the great importance of 'vitamin G' in poultry feeding.

It was found by Kline, Keenan, Elvehjem and Hart ('32, '33)[2] that the pellagra-like syndrome discovered by Ringrose, Norris and Heuser ('31)[1] could be greatly intensified by feeding chicks a diet which had been subjected to dry heat treatment. The diet consisted of yellow corn meal, wheat middlings and commercial casein heated in an air oven at 100° for 144 hours or 120° for 24 hours (Keenan, Kline, Elvehjem and Hart, '35)[4] and supplemented with minerals and cod liver oil. This discovery by the Wisconsin workers led to rapid advance in the study of the vitamin G group of factors with chicks. Elvehjem and Koehn ('35)[1] found that the syndrome produced by the heated diet was cured by the filtrate from an aqueous extract of liver after flavin had been removed by adsorption on fuller's earth. The addition of flavin to the basal diet intensified the symptoms. It was proposed to apply the name 'vitamin B_2' to the factor in the filtrate rather than, as Kuhn and others have, to flavin. The results of Elvehjem and Koehn were confirmed by Lepkovsky and Jukes ('35),[1] who noted that basal diets similar to that employed

by Elvehjem and Koehn, or by Ringrose, Norris and Heuser, tended to be deficient in flavin, and that flavin had a marked growth promoting action on the chick when fed with a diet containing an adequate supply of other vitamins, including the factor in the filtrate.

Thus two factors in the vitamin G group have been shown to be essential for chicks. Further work will be needed to establish the exact role of each of these factors in poultry nutrition, since many experiments on 'vitamin G' in poultry feeding undoubtedly dealt with a mixture of the two factors, both of which are strongly growth promoting. The factors are: 1) flavin, 2) the factor curing the dermatitis produced by the heated diet, called 'vitamin B_2' by Elvehjem and co-workers; and the 'filtrate-factor' by Lepkovsky and Jukes. It is not advisable to apply the term 'chick anti-dermatitis factor' to the filtrate factor, since Lease and Parsons ('34) showed that there are two 'chick anti-dermatitis factors.'

The nomenclature of vitamin $G(B_2)$ is in a confusing state. Elvehjem and Koehn apply the term 'vitamin B_2' to the factor curing the heated diet syndrome in chicks. Strong support is lent to this terminology by their recent experiments (Koehn and Elvehjem, '36)[2] which showed that concentrates of the factor would cure blacktongue in dogs, regarded as the analogue of pellagra in man. Many workers, on the other hand, refer to lactoflavin as 'vitamin G' on the basis of the large number of 'vitamin G' studies which have been carried out in recent years with chicks and rats in which lactoflavin was probably the primary factor involved. Yet another attitude is adopted by György ('35)[1] and others who speak of a group of factors as the 'vitamin B_2 complex,' and undoubtedly the 'vitamin $G(B_2)$' assays of recent years were measurements of a mixture of factors. These differences of terminology could probably be easily abolished by a committee on nomenclature.

Lepkovsky and Jukes ('36)[2] found that chicks on a diet low in flavin but high in the filtrate factor grew very slowly and developed diarrhea. No dermatitis was observed, and growth of feathers seemed unimpaired. The symptoms were

prevented and growth was restored by the addition of crystalline lactoflavin or hepatoflavin to the diet. The great importance of flavin is also indicated in reports by Bethke, Record and Wilder ('36 b) and Davis, Norris and Heuser ('36). The first-named workers found that pure flavin increased growth of chicks and prevented the occurrence of a leg disorder (a flexion of the toes with medial rotation of the hock) first described by Norris, Heuser and Wilgus ('29, '30).[1] Davis, Norris and Heuser stated that feeding 600γ of 'purified extract of vitamin G' per hen per week restored normal hatchability to eggs produced on a basal ration deficient in 'vitamin G.'

Findings at Cornell were summarized and analyzed in a recent bulletin by Norris, Wilgus, Ringrose, Heiman and Heuser ('36).[1] A chick unit of 'vitamin G' approximately equal to 1 microgram of flavin was proposed, and many feeding stuffs were quantitatively assayed for 'vitamin G.' It was found that chicks needed approximately 290 units of 'vitamin G' per 100 gm. of feed in order to attain normal weight at 8 weeks of age. Hens required about 230 units per 100 gm. of feed in a breeding diet, but 130 units sufficed for normal egg production. In the writer's experience, diets such as the chick basal diet used for vitamin G assay are deficient not only in flavin, but also in the strongly growth-promoting filtrate factor. The vitamin G standard was dried pork liver, which is rich in both factors. Hence the 'vitamin G' unit was probably based, not on flavin alone, but on a composite growth-promoting effect of flavin and the filtrate factor.

The properties of the filtrate factor ('vitamin B_2' of Elvehjem) were studied by Elvehjem and Koehn ('35)[1] and by Lepkovsky and Jukes ('36).[2] It was reported to be colorless, soluble in water and moist, weakly polar solvents; but insoluble in ether. It was found not to be adsorbed on fullers' earth, charcoal or lead sulfide. Exposure to visible light did not destroy the factor. It resisted treatment with several oxidizing and reducing agents. In a later report (Jukes, '37)[2] the factor was stated to be soluble in 99.5% ethanol and in 25%

phosphotungstic acid and was not destroyed by benzoyl chloride.

Jukes and Lepkovsky ('36)[2] described a method for quantitative assay of the factor, using a heated diet to which had been added a fuller's earth adsorbate of whey to supply lactoflavin, and reported the distribution of the factor in some feeding stuffs. Growth-promotion by the factor was found to increase in linear proportion to the amount of the factor which was fed, until a maximal growth was reached. The daily dose of the factor which just sufficed to produce this maximal growth in a 3-week-old chick was defined (Jukes, '37)[2] as 10 units. Since the food intake of such a chick is about 10 gm., the requirement for growth is about 100 units per 100 gm. of feed. Cane molasses appeared to be the cheapest concentrated source. Further assays (Jukes, '37)[2] indicated marked differences between the distribution of the filtrate factor and the human P-P factor in natural foods. Egg yolk was quite rich in the filtrate factor.

Curiously enough, lactoflavin, while not preventing or curing dermatitis in chicks, appears to be the anti-dermatitis vitamin of the turkey (Lepkovsky and Jukes, '36).[2]

No results have yet been reported on the effect on chicks of a deficiency of 'vitamin B_6' (György, '35 b)[1] preventing an acute dermatitis in rats ('factor 1' of Lepkovsky, Jukes and Krause, '36).[2] 'Vitamin B_6' is quite distinct from the 'filtrate factor.' The differences are summarized in table 1.

5. Other factors

Kline, Elvehjem, Keenan and Hart ('34)[4] reported a growth factor for chicks present in water-extracted liver residue. The factor was very stable to heat, since it resisted autoclaving at its natural pH for 10 hours, and was not destroyed by dry heating at 120° for 6 days. It was destroyed in liver residue by autoclaving at pH 9 for 5 hours at 15 pounds pressure. It was insoluble in water until the liver had been mildly hydrolyzed with acid. After this it could be partially extracted with normal butyl alcohol. The factor was later

found to be arginine (Arnold, Kline, Elvehjem and Hart, '36). This observation is very interesting in view of the fact that the basal diet was apparently well supplied with arginine, and addition of casein was ineffective.

Nestler, Byerly, Ellis and Titus ('36) fed hens a basal feed mixture containing "at least enough vitamin G to meet the minimum requirements of chickens for hatchability." The

TABLE 1

Differences between 'vitamin B_6' and the 'filtrate factor' (chick anti-dermatitis vitamin)

'Vitamin B_6' rat antidermatitis factor	'Filtrate factor,' chick anti-dermatitis factor, 'vitamin B_2' of Elvehjem and Koehn ('35)
Cures characteristic dermatitis in rats (by definition)	Does not cure rat dermatitis [a]
Does not cure chick dermatitis produced by heated diet [a]	Cures chick dermatitis produced by heated diet (by definition)
Adsorbed by fuller's earth in acid solution [a,b]	Not adsorbed by fuller's earth [a,c]
Destroyed by benzoyl chloride [b]	Resistant to benzoyl chloride [d]
Destroyed by visible light [e]	Not destroyed by visible light [c]
Extracted from feeding stuffs by water with much difficulty [b]	Readily extracted from feeding stuffs by water [d]
Precipitated by phosphotungstic acid [b]	Not precipitated by phosphotungstic acid [d]

Marked differences in natural distribution [d,f,g]

[a] Lepkovsky, Jukes and Krause ('36).[2]
[b] Birch and György ('36).[2]
[c] Elvehjem and Koehn ('35).[1]
[d] Jukes ('37).[2]
[e] György ('35).
[f] Birch, György and Harris ('35).
[g] Jukes and Lepkovsky ('36).[2]

ration lacked a factor necessary for high hatchability which was relatively abundant in dried pork liver meal. Hatchability was also improved by allowing the birds to range on grass and some improvement was effected by addition of a mixture of meat meal, fish meal and buttermilk. Dried whey produced little improvement. The nature of the factor, and its possible relation to other dietary essentials, including amino-acids, is not clear from the results.

The egg white syndrome. The work of Lease and Parsons ('34) showed that the symptoms produced in chicks by adding fermented egg white to a complete diet were cured, not by liver extract, but by liver residue. The symptoms simulated the dermatitis produced by feeding a heated diet of natural foodstuffs. The heated diet dermatitis, however, was cured, not by liver residue, but by liver extract. Heiman ('35)[1] found that dried skim milk or dried whey, at levels as high as 20%, did not alter the severity of the pellagra-like syndrome produced by feeding a diet containing dried egg white. Dried skim milk and dried whey are fairly good sources of the 'filtrate factor' (Jukes and Lepkovsky, '36)[2] curing the heated diet dermatitis. Hence the egg white syndrome appears to have an entirely different etiology from the heated diet dermatitis in spite of the similarity in appearance of the two conditions.

6. Slipped tendon ('perosis') and manganese

The hock deformity known as 'slipped tendon' or 'perosis' is associated with high levels of phosphorus in the diet of growing chicks (Milby, '33).[6] Hogan, Shrewsbury and Kempster ('28) found that the bones in chicks with slipped tendons were of normal ash content and that the percentages of calcium and inorganic phosphorus in the blood were normal, thus sharply differentiating the condition from rickets. It was noted by Card ('29, '30) that excessive quantities of bone meal in the ration tended to cause slipped tendons.

The discovery of a separate preventive factor was first announced by Lee ('29), who reported that a 'leg weakness,' not prevented by cod liver oil or yeast, was largely prevented by the addition of 15% of either bran or middlings to the ration in addition to the use of cod liver oil. Oats or oat feed were reported by Hunter, Dutcher and Knandel ('30–'31),[6] and rice bran by Titus ('30–'31), to possess beneficial properties for the prevention of slipped tendons. Titus ('32)[6] suggested that the preventive factor possibly belonged to the vitamin B complex. Sherwood and Couch ('33) reported

protective results with rice bran and oat groats, and found that the preventive factor was also present in wheat gray shorts. Wilgus, Norris and Heuser ('35) determined the perosis-preventing properties of certain feed stuffs, and found the relative effectiveness to be as follows: Wheat germ, 100; wheat standard middlings, 65; red dog flour, 50; wheat bran, 40; ground oats, 30; hard wheat, 5; and soy bean oil meal, 0. The effectiveness of ground yellow corn and alfalfa meal could not be determined in this manner, but, by indirect means, both of them were found to have a slight preventive action. It was found by Serfontein and Payne ('34) that a tendency toward perosis could be inherited. The sum of evidence made it apparent that several conditions contributed to the etiology of perosis.

Recently Wilgus, Norris and Heuser ('36) have announced that either 0.0035 or 0.0160% of manganese in the diet is quite effective in preventing perosis at levels of calcium of 1.0 and 1.2% and of phosphorus of 0.8 and 1.2%. Aluminum and zinc possessed some preventive action. The perosis preventing property of feed stuffs was roughly related to their manganese content. Almost simultaneously, Sherwood and Fraps ('36) reported that the protective factor in wheat gray shorts is one or more of the mineral constituents of the ash.

PART B. RECENT STUDIES WITH VITAMINS A, B(B₁), D AND E

1. *Vitamin A*

The importance of vitamin A in poultry nutrition is very great. The subject was ably reviewed by Cruickshank ('35–'36).[6] Cruickshank's review included a description of the pathological symptoms of vitamin A deficiency in chickens. Our knowledge of the pathology has been since supplemented by Hinshaw and Lloyd ('34)[5] who made a comparative study of vitamin A deficiency in chicks and turkeys.

Recent work, which will be dealt with in this review, deals principally with quantitative aspects of the vitamin A requirement of chickens.

a. Vitamin A requirements of the growing chick. The chick is hatched with a reserve of vitamin A in the yolk sac and the liver, and estimations of the vitamin A requirement of growing chicks are hence often based on studies of birds at 8 weeks of age, thus allowing time for the reserves to become exhausted.

Frohring and Wyeno ('34)[5] found, by a rather involved calculation, that the minimum vitamin A requirement of an 8-week-old chick was about 92 international units per day when fed as a daily dose of carotene in cottonseed oil. A similar result was obtained with cod liver oil. Record, Bethke and Wilder ('35)[5] reported that 50 to 100 micrograms of carotene (80 to 170 international units as beta-carotene) per 100 gm. of feed were required for normal growth to 8 weeks of age. The same amount, in terms of rat units, was required when the vitamin was supplied as cod liver oil. Wilson, Schroeder and Higgins ('36) also reported that, unit for unit, carotene and vitamin A obtained from a fish oil concentrate were utilized equally well by the chick. Ringrose and Norris ('36)[5] used carotene as a standard in a careful study of the vitamin A requirements of the growing chick. They found that 100 international units per 100 gm. of feed were just sufficient for prevention of symptoms up to 8 weeks of age but not sufficient to allow normal growth. One hundred and fifty units was practically as satisfactory for growth as higher levels. Yellow corn was found to furnish about 7 units of vitamin A per gram. Smith ('33)[5] found that between 12.5 and 25% of yellow corn supplied the vitamin A requirement. This would correspond to between 88 and 175 international units of vitamin A per 100 gm. of feed if the corn contained 7 units per gram. Wilson, Schroeder and Higgins ('36) stated that 264 international units per 100 gm. of feed were adequate to promote satisfactory growth. The minimum requirements were not stated.

Biely and Chalmers ('36 a) fed vitamin A to chicks by pipette in weekly doses. By means of this technic, they found that the weekly administration of 525 units of vitamin A was

sufficient to ensure normal growth and protection against deficiency symptoms up to 8 weeks of age. The method is not easily comparable with normal feeding practice, since the dose did not vary with the steadily increasing food intake of the growing chick. Hence a chick might tend to store vitamin A at the beginning of the experiment, and protect itself from deficiency in the later stages by drawing on its store. The same authors ('36 b) reported that there were indications that sardine oil (pilchard oil) contained some substance which chicks, but not rats, could utilize as a source of vitamin A, but a contrary opinion was expressed by Milne ('36), who found no indications that pilchard oil (sardine oil) contained any growth promoting factor which was not vitamin A.

b. Vitamin A requirements of laying hens. The vitamin A requirements for egg production have been extensively studied by Sherwood and Fraps ('33, '34, '35).[5, 6] They express their results in Sherman-Munsell units, which have been converted to international units in this review. In their first report it was noted that a mash containing 20% of ground yellow corn, supplemented with yellow corn as scratch grain, failed to supply the requirements, since the vitamin A content of the yolk decreased from 28 units to from 7 to 11 units during the experiment. It was calculated that 6.3 units in the feed were required to produce 1 unit in the egg, and the maintenance requirement was estimated at 150 units per day. The use of dried alfalfa was stated to be insufficient to supply the requirements, and green feed was recommended in addition. In 1934 the same authors noted a decline in the vitamin A content of the egg yolk during the laying season even when the hens received 630 units per day. The hens receiving 630 units, supplied as dried alfalfa, laid about 15% more eggs than hens receiving 320 and 480 units. However, it appears to the writer that the rations supplying the lower levels were probably deficient in lactoflavin. Four units of vitamin A in the feed were required to produce 1 unit in the egg. Green feed was again recommended as being necessary to supplement the ration. However, it seems that the alfalfa meal

used was of distinctly low potency (110 units per gram). Sherwood and Fraps ('35) continued the investigation and found that the vitamin A requirements for the formation of feathers seemed to be as high as the requirements for egg production, since molting hens did not store the vitamin even when fed liberal amounts. The authors estimated that 340 units per day, or about 430 units per 100 gm. of feed were required to keep the hens in good health and maintain a good production of eggs. Once again, it appears that the ration was somewhat low in lactoflavin.

Russell and co-workers ('36) found that increasing the vitamin A content of a ration which contained 485 units per 100 gm. did not improve egg production and hatchability and did not reduce mortality. It was concluded that the usual type of laying ration, containing yellow corn, alfalfa and fish liver oil, supplied sufficient vitamin A.

2. Vitamin B (B₁)

Chickens were the first animals to be used in experimental vitamin B studies. In spite of this, very little is known about the quantitative vitamin B requirements of the chick. This lack of information may be traced to two causes; first, it is difficult to devise a vitamin B-free ration for chicks which supplies all other essentials, second, lack of interest has been caused by the fact that practical poultry rations apparently more than sufficiently meet the vitamin B requirements of the chick.

Kline, Keenan, Elvehjem and Hart ('32–'33)[2] found that chicks developed acute symptoms of polyneuritis when fed an autoclaved ration consisting of yellow corn, 58%; wheat middlings, 25%; casein, 12%; minerals and cod liver oil, 5%. When 16% of the unheated ration was added to the autoclaved ration, some protection was afforded, and when 32% was added, the chicks grew 'normally.' Hence it appears that the unheated ration, if fed alone, would have supplied at least three times the necessary amount of vitamin B(B₁) for chicks.

3. Vitamin D

The discovery 15 years ago of the need of poultry for the antirachitic factor has had a profound influence on feeding practice. Space does not permit a discussion of more than a few of the recent investigations of vitamin D which have been made with chicks.

a. The forms of vitamin D and their varying effectiveness for chicks and rats. Following the original discovery of Carrick (Bills, '35),[3] it was clearly shown by Massengale and Nussmeier ('30),[3] Mussehl and Ackerson ('29, '30)[6] and Hess and Supplee ('29, '30)[6] that cod liver oil, rat unit for rat unit, is superior to irradiated ergosterol as a source of vitamin D for chicks. Subsequent investigators have repeatedly confirmed this finding. The difference is not due to poorer uptake of irradiated ergosterol, because Klein and Russell ('31)[6] found that of the total amount of vitamin D fed as irradiated ergosterol to chicks during the first 4 weeks of life, 26.5% of the rat units consumed were recovered in the droppings, and when cod liver oil was fed instead, the recovery was 43.1%. Russell, Taylor and Wilcox ('32–'33) also noted that essentially the same bone ash percentages were obtained by administering vitamin D to chicks by injection as when it was fed by capsule. Irradiated ergosterol and cod liver oil were both used. The relative superiority of 1 rat unit of cod liver oil to 1 rat unit of irradiated ergosterol for the chick has been found to range from fifteen- to twentyfold (Bethke, Record and Kennard, '33)[3] to 144- to 192-fold (Russell, Taylor and Wilcox, '32–'33). Waddell ('34) clearly demonstrated that irradiated cholesterol is much more efficacious, rat unit for rat unit, than irradiated ergosterol in preventing rickets in chicks. This finding by Waddell immediately draws attention to the possibility that other sterols were contaminants of certain of the ergosterol preparations used in making comparisons between rats and chicks, i.e., that the difference, e.g., between the results of Bethke, Record and Kennard ('33)[3] and those of Russell, Taylor and Wilcox ('34), alluded to above, may be due to varying amounts of traces of other sterols

with pro-vitamin D activity in the various samples of ergosterol used. Opposition to this suggestion is found in an article by Bills, Massengale, McDonald and Wirick ('35).[3] These workers standardized with rats seven specimens of irradiated partially purified ergosterol, and found the specimens not to differ, one from another, in their effects on the blood and bones of chickens. They found 1 rat unit of vitamin D in cod liver oil to be fifty times as effective for the chick as 1 rat unit of vitamin D in irradiated ergosterol. Previously they had found a difference of 100 times. They comment that this difference is greatly affected by varying experimental conditions.

Bills ('35)[3] has discussed further the standardization of the rat: chick comparison. He draws attention to the importance of the 'response curve' for chicks, obtained by plotting percentage bone ash against vitamin unitage. The 'response curve' has different shapes for different sources of vitamin D, and allowance for this fact must be made in comparing various forms of vitamin D. Presumably it would be of advantage to research workers to use a more standardized chick test for the estimation of vitamin D in order to facilitate the comparison of work carried out by different laboratories. Bills ('35)[3] states further that "unless the test period is extended to longer than 4 weeks one cannot produce quite as hard a bone in chickens with irradiated ergosterol as one can with cod liver oil, no matter how large the dosage given." Massengale and Bills ('36)[3] have also discussed refinements in the chick test.

Certain experiments have indicated that some form or forms of vitamin D are even more effective for chicks, rat unit for rat unit, than the vitamin D of cod liver oil. Bills, Massengale, Imboden and Hall ('36) reported that the vitamin D of white sea bass liver oil was 2.6 times as effective for chicks, rat unit for rat unit, as that of cod liver oil. The latter had the same degree of relative effectiveness as irradiated 7-dehydro-cholesterol. Hathaway and Lobb ('36) concluded that the vitamin D of irradiated crude cholesterol was more effective

for chicks, rat unit for rat unit, than that of cod liver oil. Koch and Koch ('36), from spectroscopic studies, inferred that 7-dehydrocholesterol and heated cholesterol were different forms of pro-vitamin D. From comparative experiments with rats and chicks, they concluded that corn phytosterol and ergosterol were two additional forms of the pro-vitamin. Recent conceptions of the structural formulas of certain forms of vitamin D and its precursors were reviewed by Bacharach ('36) and Grab ('36).

A comparison of several irradiated oils was made by Haman and Steenbock ('36). The vitamin D activity of cod liver oil for the chick was found not to be increased by irradiation. Comparative studies showed that the provitamin D of animal fats gave rise to a more effective form of the vitamin, rat unit for rat unit, than the provitamin D of plant oils, when animals fats and plant oils were irradiated and fed to chicks.

The comparative antirachitic efficacy for rats and chicks of several forms of vitamin D was studied by Grab ('36). A tuna liver oil concentrate was used as the standard rather than cod liver oil, and Bills, Massengale, Imboden and Hall ('36) have shown that the efficacy ratio of tuna oils is determined by the species of tuna. Hence it is difficult to compare the results of Grab ('36) directly with those of other investigators. Grab found the efficacy for *rats,* chick unit for chick unit, compared with the efficacy of vitamin D of tuna liver oil concentrate to be for calciferol (vitamin D_2) 32; vitamin D_3, prepared from irradiated 7-dehydrocholesterol 0.65; vitamin D_3, prepared from tuna liver oil 0.60; irradiated 22-dihydro-ergosterol 2.5 to 10; irradiated 7-dehydrositosterol more than 13. Experiments with 7-dehydrositosterol and 7-dehydro-stigmasterol led to the conclusion that 29-carbon-atom sterols were too weak in provitamin D activity to be of any importance either to rats or chicks as sources of the vitamin.

Table 2 summarizes some recent studies on vitamin D. It has not been attempted to list all of the various forms of vitamin D in the table, and certain sources of the vitamin named in the table are probably mixtures, but the tabulated

TABLE 2

Certain sources and precursors of vitamin D and their comparative effectiveness for chicks and rats

SOURCE OF VITAMIN D	PRECURSOR	PROBABLE FORM	EFFECTIVENESS FOR RATS	EFFICACY QUOTIENT, I.E., THE APPROXIMATE EFFECTIVENESS OF 1 RAT UNIT FOR CHICKS AS COMPARED WITH 1 RAT UNIT OF COD LIVER OIL
Calciferol	Ergosterol	Vitamin D_2	40,000 I.U. per mg. [a]	0.02 [b]
Calcium cholesterilene sulfonate	Cholesterol	?		>1 [i]
Irradiation product	Unheated crude cholesterol	?	+++	>1 [c]
Irradiation product	Unheated purified cholesterol	?	+	<1 [c]
Irradiation product	Heated purified cholesterol	?	+++	1 [c]
Irradiation product	7-dehydrocholesterol	Vitamin D_3	24,000 I.U. per mg. [a], 25,000 I.U. per mg. [d], 17,000 I.U. per mg. [j]	1 [e]
Tuna liver oil	?	Vitamin D_3	25,000 I.U. per mg. [d], 20,000 I.U. per mg. [j]	See text
Irradiation product	7-dehydrositosterol	?	1/40 activity of irradiated ergosterol [f] 500 I.U. per mg. [j]	See text
Irradiation product	22-dihydroergosterol	?	2000 I.U. per mg. [j], 2500–3100 I.U. per mg. [h]	<1, but more effective than irradiated ergosterol [h]
Irradiation product	Corn phytosterol	?	+	Three times as effective as viosterol [g]
Cod liver oil	?	?	++	1
White sea bass liver oil	?	?	++	2.6 [e]
Albacore liver oil	?	?	+	0.9 [e]

[a] Windaus, Schenck and Werder ('36).
[b] Bills, Massengale, McDonald and Wirick ('35).
[c] Hathaway and Lobb ('36).
[d] Brockmann ('36).
[e] Bills, Massengale, Imboden and Hall ('36).
[f] Wünderlich ('36).
[g] Koch and Koch ('36).
[h] McDonald ('36).
[i] Yoder, Thomas and Lyons ('35).
[j] Grab ('36).

findings are all illustrative of the role of the chick test in
the elucidation of vitamin D.

b. *The vitamin D requirements of growing chicks.* The
preceding section has drawn attention to differences in the
effectiveness of various forms of vitamin D for the chicks.
These differences make it evident that quantitative estimates
of the requirements of chicks for vitamin D are dependent

TABLE 3

*The vitamin D requirements of growing chicks as determined by means of
cod liver oil*

INVESTIGATORS	INTERNATIONAL UNITS REQUIRED PER 100 GM. OF FEED	CRITERIA
Bethke, Record and Kennard ('33)	19 [a]	Normal calcification
Russell, Taylor and Wilcox ('34)	15 [b]	Normal bone ash and body weight
Carver, Robertson Brazie, Johnson and St. John ('34)[c]	17	Satisfactory calcification and growth
Murphy, Hunter and Knandel ('36)	19	Bone histology, blood serum calcium, growth
Couch, Fraps and Sherwood ('35)	12.3 for maximum growth 3.1 for prevention of rickets	
Hathaway and Lobb ('36)	27 adequate 13.5 slightly inadequate	Weight gain Bone ash

[a] 7 Steenbock units.
[b] 5.5 Steenbock units.

upon the form of the vitamin used. If we assume that cod
liver oil contains only one form of the vitamin or a uniform
mixture of two or more forms, it is possible to compare the
findings of different workers (table 3). Allowances must be
made for genetic differences, various criteria used for esti-
mating rickets, differences in feed consumption, and differ-
ences in calcium and phosphorus content of the various rations
used.

Among practical sources of vitamin D for chicks, sardine oil (pilchard oil) has been found to be as effective as cod liver oil (Biely and Palmer, '33; Tepper, '35).

c. *The vitamin D requirements of laying hens.* Carver and co-workers ('34)[6] found that laying hens in confinement without sunlight required 67 international units of vitamin D (cod liver oil) per 100 gm. of feed to secure satisfactory egg production and 'egg quality,' and 135 units to ensure maximum hatchability. Murphy, Hunter and Knandel ('36)[3] found that 78 international (U.S.P.) units of vitamin D (cod liver oil) per 100 gm. of feed for laying pullets, confined without access to sunlight, produced satisfactory results in maintenance of body weight, egg production, egg size, quality of egg shell, and hatchability. Fifty-eight units were insufficient.

d. *Ultra-violet irradiation as a source of vitamin D for chicks.* The vitamin D requirements of chickens are supplied with remarkable ease by exposure to ultra-violet irradiation. The precursor in the surface of the bird's body has not been identified. The interesting claim has been made by Hou ('29) that the sebaceous secretion of the preen gland is spread by birds over their plumage and then reingested following exposure to sunlight to serve as the principal source of vitamin D for birds. However, Knowles, Hart and Halpin ('35) found that removal of the preen gland from chicks had no depressing effect upon growth or skeletal development when the sole source of vitamin D was irradiation from a quartz mercury vapor lamp.

It was found by Maughan ('28) that the region of the spectrum most effective in curing rickets in chicks was in the neighborhood of 2965Å. Wave lengths of 3130 and longer were ineffective. Wave lengths shorter than 2894 seemed to have little antirachitic power, but this might have been due to the absorption of the shorter rays by the dead cell layer on the surface of the skin. Maughan also noted the great efficiency of the mercury vapor lamp in curing rickets in chicks; an exposure of 1 minute per day at a distance of about 56 cm. brought about 95% recovery in 28 days. Scott, Hart

and Halpin ('29) found that irradiation with a quartz mercury vapor lamp once a week for a 3-minute period at a distance of 56 cm. was sufficient to prevent rickets in chicks.

Light is similarly effective for supplying the vitamin D requirement of laying hens. Carver and co-workers ('34)[6] found that, under the conditions of the experiment, sunshine (sun porches) was sufficient as a sole source of vitamin D to provide for growth of pullets to 24 weeks, and for egg production and hatchability during the summer months. Winter egg production and hatchability were improved by additional vitamin D in the form of cod liver oil. Murphy, Hunter and Knandel ('36)[3] reported that, for the conditions experienced, Leghorn pullets given access to range throughout the entire laying period may secure sufficient vitamin D by irradiation from direct sunshine for satisfactory performance. Body weight, percentage egg production, egg weight, percentage shell of egg, percentage ash of shell, blood serum calcium, and hatchability of fertile eggs were the criteria of performance.

No results have been published which give the comparative efficacy for chicks, rat unit for rat unit, of the vitamin D of cod liver oil and the vitamin D of egg yolk from hens which have received ultra-violet light as the sole source of the antirachitic factor. Such a study might give valuable information on the form of vitamin D which is synthesized with such apparent ease in the skin of the hen.

4. Vitamin E

Ordinary poultry rations contain comparatively large amounts of the germs of grains, and are usually supplemented with fresh or dried greens. Hence it is generally assumed that such rations supply ample amounts of vitamin E. Support for this assumption is found in the results of Card ('28, '29),[6] who observed that the addition of wheat germ oil to a ration containing corn, wheat bran, wheat middlings, meat scrap and salt did not improve fertility or hatchability.

The discovery by Waddell and Steenbock ('28) of the ferric chloride treatment for destruction of vitamin E in natural food stuffs, facilitated studies of the vitamin E requirements of poultry. Card, Mitchell and Hamilton ('30) fed a ration treated in this manner to pullets from 8 weeks of age to maturity. The pullets were mated with males which received vitamin E. Of 354 eggs laid in a period of 53 days, 317 were fertile as shown by candling, but only forty-one developed beyond the ninth day of incubation, and none hatched. Pathological conditions in the embryos in this experiment were described by Adamstone ('31). It is impossible to say whether or not the pathology was influenced by a deficiency of the anti-hemorrhagic vitamin. The administration of 0.5 cc. of wheat germ oil daily to the pullets brought the hatchability to normal. Discontinuation of wheat germ oil feeding resulted in immediate falling off of hatchability. The results seemed to indicate clearly the necessity of vitamin E in the diet of the hen for hatchability, although egg production and fertility were apparently not greatly affected by vitamin E deficiency. The effects of the same diet on male chickens were studied by Adamstone and Card ('34). After 1 year on the vitamin E-free diet all of the males were capable of fertilizing ova, but after 2 years some of the males were sterile. Histological studies were made and some degenerative changes were revealed. The authors concluded that the testis of the fowl was extremely resistant to vitamin E deficiency.

Barnum ('35) found by means of rat tests that eggs were low in vitamin E if laid by hens on diets designed to be deficient in this vitamin. It was also noted that the eggs were of low hatchability. Hatchability was improved by the addition of 15% of wheat germ to the diet of the hen, but no such improvement was produced by adding lettuce. It has since become apparent from the results of Bethke, Record and Kennard ('36) that wheat germ may contain a factor, not vitamin E, which improves hatchability. The results of Barnum ('35) do not indicate any possibility of a deficiency of vitamin E in the usual poultry rations.

A report by Ender ('35) on improvement of hatchability by wheat germ oil is difficult to interpret because the experiment was not controlled with respect to seasonal variation of hatchability.

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THE IDENTITY OF FLAVIN WITH THE CATARACT-PREVENTIVE FACTOR [1]

PAUL L. DAY, WILLIAM J. DARBY AND WILLIAM C. LANGSTON

School of Medicine, University of Arkansas, Little Rock

THREE FIGURES

Evidence from several laboratories indicates that there are at least two heat-stable vitamins of the B complex essential to the nutrition of the rat (Chick, et al., '35; György, '34, '35; Harris, '35; Hogan and Richardson, '36; Bender, et al., '36; Dann, '36; Copping, '36). One of these vitamins, lactoflavin (d-riboflavin), has been isolated, chemically identified, and synthesized (von Euler, '36). The other known factor can at present be identified only by the symptoms that its lack produces in experimental animals. A deficiency of this factor (called vitamin B_6 by many writers) produces in rats a marked inflammation and edema of the paws, ears and nose. The syndrome has been termed 'rat pellagra' by several investigators, 'specific dermatitis' by György ('34, '35), 'florid dermatitis' by Chick, Copping and Edgar ('35), and 'rat acrodynia' by Birch, György and Harris ('35).

A deficiency of flavin produces in young rats a denuded condition, called a 'skin affection' by Chick, Copping and Edgar ('35), and 'non-specific' lesions by György ('34, '35). Since there is usually no inflammation of the skin accompanying this condition, it is not, strictly speaking, a dermatitis. As a loss of hair is the most characteristic feature of the condition, the term 'alopecia' probably describes it as adequately as any single word can do.

[1] Research paper no. 505, journal series, University of Arkansas.

In a series of papers from this laboratory, embracing work extended over a period of more than 6 years, we have reported the consistent appearance of cataract in rats as a result of 'vitamin G' deficiency (Day, et al., '31; Langston and Day, '33, '34; Day, '34, '34 a; Day and Langston, '34; Day and Darby, '36). This has been corroborated by enough widely separated laboratories (O'Brien, '32; Yudkin, '33; Guha, '35; Bourne and Pyke, '35) to render it quite unlikely that the cataract is due to any peculiarity of our experimental technic or strain of animals. Some of these investigators have suggested, however, that cataract may result from a deficiency of some yet unidentified factor.

The availability of pure lactoflavin has made it possible for us to determine whether or not this substance would prevent cataract in our experimental vitamin G-deficient animals. Experiments have been made in which our deficient diet was supplemented with various amounts of pure flavin. The results of these experiments constitute this report.

EXPERIMENTAL METHODS AND RESULTS

The cataract-preventive action of pure lactoflavin. The vitamin G-deficient diet (no. 625) used in the experiment had the following composition: casein, vitamin-free, Labco brand (Suplee et al., '36), 18%; cod liver oil,[2] 2%; butter fat, 8%; salt mixture, Osborne and Mendel ('19), 4%; cornstarch, 68%. The cornstarch carried an extract of rice polish prepared with 80% ethyl alcohol (Day and Langston, '34) evaporated down on the starch and incorporated in the diet so that each 100 gm. of the diet contained the extract of 25 gm. of rice polish. Our method of caging, care and selection of animals has been described (Day and Darby, '36).

Two samples of lactoflavin were used in the feeding experiments. The Winthrop Chemical Company supplied us with some synthetic lactoflavin, and Dr. S. Lepkovsky kindly sent us a small amount of natural lactoflavin that had been prepared in his laboratory. Dr. O. W. Barlow of the Winthrop

[2] Supplied by the E. L. Patch Company, Boston.

Chemical Company has given us the following statement of properties for their synthetic product: melting point, 292°C.—decomposition; melting point of tetra-acetyl deriva-tive, 242°C.; optical rotation in 0.05 N NaOH, $[\alpha]_D^{20°} = -110°$ $\pm$ 5°; optical rotation in 0.05 N NaOH + half saturated borax, $[\alpha]_D^{20°} = 365° \pm 10°$. These properties are in good agreement with the physical constants reported in the literature for
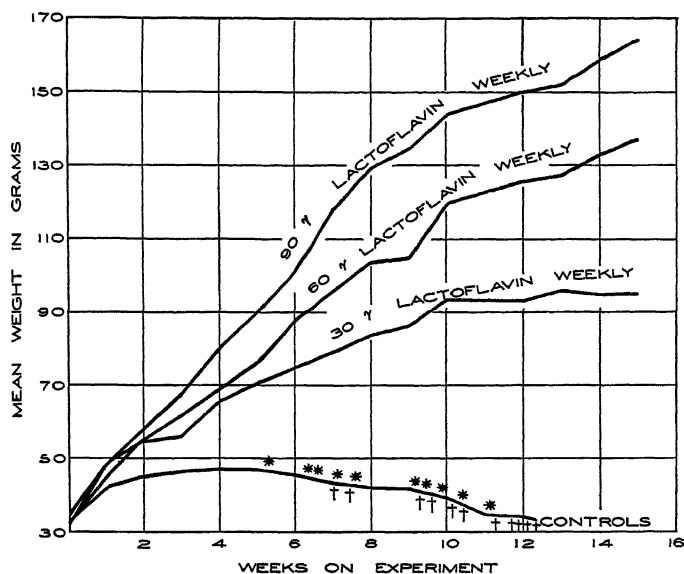
Fig. 1   Composite weight curves of rats receiving the flavin-deficient diet no. 625 (controls), and of rats receiving the same diet supplemented with pure lacto-flavin. The asterisks mark the time of appearance of cataract, and daggers mark the death of individual animals. None of the rats receiving flavin exhibited evidence of cataract.

lactoflavin (von Euler, '36). The lactoflavin was fed in weekly doses of 30, 60 and 90 micrograms, to groups of six rats on each level of feeding. Eleven control rats, littermates of the eighteen rats receiving flavin, were given the deficient diet only. Composite weight curves for the eleven control animals and for the rats receiving synthetic flavin at the three levels of feeding are shown in figure 1, and photographs of typical animals are shown in figure 2.

One of the eleven control rats died on the forty-ninth day without exhibiting cataract. The remaining ten rats all showed cataract between the thirty-seventh and seventy-eighth days, as seen with the ophthalmoscope; the average time of appearance was 58 days. Four of the eleven rats exhibited gross, mature cataract, readily evident with the naked eye by the most casual observation. Although weekly ophthalmoscopic examinations are a part of our regular routine, the use of the ophthalmoscope is not essential for the detection of

Fig. 2   Photographs of typical experimental animals. Above—negative control that received the flavin-deficient diet only. Alopecia of the face and cataract are evident. Below—rat which received the flavin-deficient diet supplemented with 90 micrograms of flavin weekly. There was no evidence of alopecia or cataract. The animals were photographed on the seventy-fifth day of experiment, at which time they weighed 35 and 150 gm. respectively.

most cases of cataract. After dilation of the pupil with 0.5% atropine sulphate solution, most cases of early cataract may be detected without the use of the ophthalmoscope, if the eyes of such animals are observed in a partially darkened room with the aid of a small pocket flash lamp. The beam is directed into the eye at an oblique angle from the line of vision of the observer, and the lens opacity is seen as a snowy mass, appearing to be deep in the eye. Frequently the lens opacities are obscured by dense opacties in the cornea (keratitis); such

keratitis is frequently transient, however, and upon disappearing renders the cataract visible. Upon proceeding to maturity the cataract may be seen even in the presence of dense keratitis.

The eleven control animals survived between 49 and 87 days, with an average survival period of 72 days. In addition to exhibiting keratitis and cataract, many of them showed more or less extensive alopecia and roughness of hair. None of them developed the inflammation and exfoliation of the paws characteristic of 'florid' dermatitis or 'rat acrodynia.'

None of the eighteen animals receiving flavin exhibited any evidence of cataract; a few rats on the lowest flavin level showed alopecia and mild keratitis. Several developed ulcers about the head, but at no time did the flavin rats show the dermatitis characteristic of vitamin B_6 deficiency. The animals receiving flavin were killed at the end of 15 weeks with the exception of one rat on each level of feeding. The animal receiving 30 micrograms weekly died during the nineteenth week without showing cataract. The other two are still alive after 25 weeks without any evidence of lenticular opacities.

Since we were primarily interested in the cataract-preventive action of the flavin rather than a quantitative determination of its growth-promoting activity, the animals were not subjected to a preliminary depletion period before the feeding of flavin was started. Hence the growth curves may not be strictly comparable to growth curves obtained by investigators employing a depletion period. The growth-promoting action of the sample of natural flavin was essentially the same as that of the synthetic material.

Differentiation of the cataract-preventive factor (flavin) from the rat dermatitis-preventive factor. As was pointed out in the introduction, evidence from several laboratories has differentiated two relatively heat-stable vitamins of the B complex required by the rat. Evidence of a similar nature has been obtained in this laboratory, and since it also differentiates the cataract-preventive factor from the rat dermatitis-preventive vitamin, it is pertinent to the present discussion.

Four groups of rats were given a basal diet deficient in all the B vitamins (diet 625 without the rice polish extract) supplemented in various ways, as follows:

Group I. Deficient diet supplemented with 60 micrograms crystalline vitamin B (Merck) weekly. This was deficient in both flavin and the dermatitis-preventive factor (vitamin B_6).

Group II. Deficient diet supplemented with rice polish extract (diet 625). This diet was deficient in flavin.
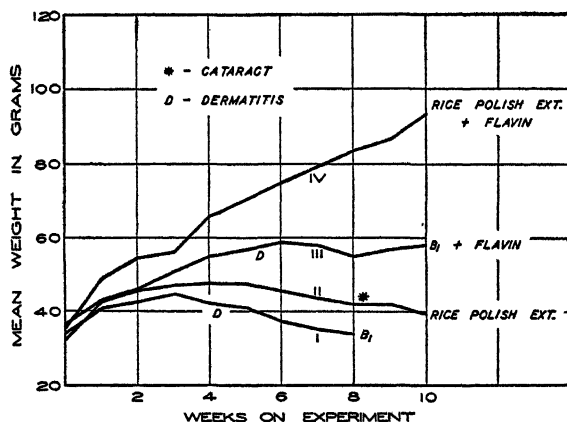
Fig. 3 Mean growth curves of groups of rats receiving a diet deficient in all B vitamins, supplemented as indicated at the ends of the curves. Rice polish ext., extract of rice polish prepared with 80% alcohol, as contained in diet 625; flavin, 30 micrograms lactoflavin weekly per rat; B_1, 60 micrograms crystalline vitamin B weekly per rat; D, dermatitis, i.e., inflammed paws, nose and ears; *, cataract. The roman numerals refer to the groups as discussed in the text.

Group III. Deficient diet supplemented with 60 micrograms crystalline vitamin B weekly and 30 micrograms lactoflavin weekly. This diet was deficient in the rat dermatitis-preventive factor (vitamin B_6).

Group IV. Deficient diet supplemented with rice polish extract and 30 micrograms lactoflavin weekly. This diet contained enough of all dietary essentials for fair growth; the growth-limiting factor was flavin.

Mean growth curves for these groups of rats are shown in figure 3; the roman numerals on the curves correspond to the four groups as indicated above. The animals in group I all

developed dermatitis (acrodynia) between the twenty-fifth and thirty-sixth days, with an average time of appearance of 29 days which is marked on the curve by the letter 'D.' Since this diet was deficient in flavin also, it would be expected that the rats receiving it would also develop cataract. Most of the rats died during the first 8 weeks of experiment, however, and only in the few remaining animals did eye changes develop. The animals in group II developed cataract, and the average time of appearance is shown on the curve by an asterisk. None of them developed the characteristic dermatitis of vitamin B_6 deficiency, although alopecia was commonly seen.

The animals in group III made only very slight growth, but all survived 10 weeks. These rats developed dermatitis but not cataract, as should be expected since they received the cataract-preventive substance (flavin), but did not receive any material containing the dermatitis-preventive vitamin. The animals in group IV did not show any evidence of either cataract or dermatitis, and made fair gains in weight. Growth was obviously limited by the rather low level of flavin feeding.

DISCUSSION AND CONCLUSIONS

The above data give evidence that our diet 625 is adequate for good growth when supplemented with pure lactoflavin. This would indicate that the rice polish extract in the diet contains considerabe amounts of vitamin B, the rat dermatitis-preventive factor (vitamin B_6), and any other of the B vitamins required for growth of the rat except flavin. Since we have not fed lactoflavin in quantities greater than 90 micrograms weekly, we do not yet know whether further increments in flavin feeding will result in further increments in growth. It may be that diet 625 does not contain enough of these various factors for normal growth, but it is clear that it contains enough of them for growth up to 10 gm. weekly. Using this diet with experiments where the growth is 10 gm. weekly or less, therefore, it is obvious that flavin is the limiting growth factor. We feel that these results justify the statement that this diet is suitable for the biological estimation

of flavin. Indeed, it is now possible to interpret all the assays for 'vitamin G' which we have reported in the last 6 years in terms of flavin, uncomplicated by the: antidermatitis factor or other growth essentials.

Since a very large percentage of the rats given our flavin-deficient diet develop cataract, and cataract may effectively be prevented with pure flavin under rigidly controlled conditions, it is apparent that flavin is a specific cataract-preventive vitamin for the rat. We find no other adequate explanation for the data reported above. It has been the practice to refer to each of the vitamins in terms of the frank disease or syndrome resulting from marked deprivation of that substance, i.e., antineuritic, antiscorbutic, antirachitic, etc. In common with this practice of referring to the vitamins in terms of the most conspicuous pathological manifestations resulting from their lack, we believe that it is entirely appropriate to refer to flavin as the cataract-preventive vitamin. In doing so there is no implication that the only function of flavin is the prevention of cataract, any more than the term antiscorbutic implies that the prevention of scurvy is the only useful function of ascorbic acid. However, cataract is a specific condition not readily confused with the pathology of any other nutritional deficiency, and, prior to the death of the experimental animal, is probably the only irreparable result of flavin deficiency.

As has already been mentioned, a number of other laboratories have also reported cataract resulting from vitamin G deficiency, but some investigators have stated that they failed to observe the condition, and many more have made no mention of it whatever. Although it would be inappropriate for us to attempt to explain why certain other laboratories have failed to observe cataract as a result of vitamin G insufficiency, there are certain quite obvious reasons that may explain some such failures. It is probable that some investigators have been dealing primarily with a deficiency of some one or more of the growth essentials other than flavin in their vitamin G experiments. Also, a very small amount of flavin will delay

the appearance of cataract for a considerable period, and greatly reduce its incidence. Consequently a small amount of flavin as a contaminant of a diet component could prevent the appearance of cataract during an ordinary experimental period. For instance, Supplee, et al. ('36) have detected the presence of flavin in certain so-called 'vitamin-free' caseins, and have stated that some of the commonly used methods for rendering casein vitamin-free do not remove the last trace of flavin. Our own experience with various casein preparations, using production of cataract as an evidence of flavin deficiency, has led us to similar conclusions. Some of the conditions influencing the appearance of cataract in flavin deficiency are being investigated, and the results will be reported later.

Our results indicate that flavin is ineffective in preventing the dermatitis of so-called 'rat pellagra' or 'rat acrodynia,' a conclusion previously reached by several other investigators. This dermatitis, characterized by inflammation and edema of the extremities, must be regarded as a specific syndrome resulting from a deficiency of some vitamin which has not yet been identified chemically. Flavin deficiency, evidenced by alopecia and cataract, can now be clearly distinguished from this other symptom-complex.

SUMMARY

Young rats given a diet deficient in all B vitamins, but supplemented with an extract of rice polish prepared with 80% ethyl alcohol, developed cataract and alopecia. Litter mates receiving the same diet, further supplemented with 30 micrograms, 60 micrograms or 90 micrograms of lactoflavin weekly, did not exhibit cataract. From this it is concluded that flavin is a specific cataract-preventive substance for the rat. Growth was roughly proportional to the amount of flavin fed, and the highest level of feeding promoted good growth. None of the animals receiving the diet, either with or without flavin supplement, developed dermatitis of the extremities. It is obvious that the rice polish extract contained considerable amounts of vitamin B, the antidermatitis

factor (vitamin B_6), and any other of the B vitamins required by the rat except flavin.

Rats given a diet deficient in all B vitamins, supplemented with crystalline vitamin B only, developed the characteristic inflammation and edema of the extremities termed 'rat pellagra,' 'florid dermatitis' or 'rat acrodynia.' Flavin failed to prevent this syndrome. Thus flavin deficiency, evidenced by alopecia and cataract, can be clearly distinguished from this other symptom-complex.

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THE PROTEIN MINIMA FOR NITROGEN EQUILI-
BRIUM WITH DIFFERENT PROTEINS [1]

DANIEL MELNICK AND GEORGE R. COWGILL

Laboratory of Physiological Chemistry, Yale University

In the present study the determinations of protein minima
for nitrogen equilibrium with different proteins were carried
out as prerequisites for the proper evaluations of these food-
stuffs with respect to their ability in promoting serum protein
regeneration (Melnick, Cowgill and Burack, '36). By such
a procedure it was possible to estimate with some degree of
accuracy what fraction of the dietary protein, ingested by the
dogs subjected to plasmapheresis, is utilized for the nitrogen
metabolism of the organism as a whole. Theoretically the
relative potencies of the various proteins for the regeneration
of serum protein should then be evidenced solely by the effects
obtained when the test increment quantities are fed. How-
ever, the determination of a protein minimum for nitrogen
equilibrium is obviously also an indication of its biological
value. For a summary of the nutritional values of many
proteins the reader is referred to the review article by Boas-
Fixsen ('34). It was hoped that an experimental determina-
tion of the minimum for nitrogen equilibrium of one of the
proteins would suffice, with the aid of values reported by
other investigators, to indicate what the corresponding

amounts should be for the other proteins. However, the relative values reported in the literature were found to be so conflicting (see table 1, Boas-Fixsen, '34) that it was impossible with any degree of accuracy to apply these data to our investigations of serum protein regeneration. It was evident, therefore, that we should determine with our own dogs, fed according to our standardized technic, the minima for nitrogen equilibrium of the proteins to be tested for their respective potencies in the regeneration of serum protein.

It is not our purpose to cite here the extensive literature concerning the biological values of proteins in view of the fact that several review articles are available (Cathcart, '21; Mitchell, '24 b; Boas-Fixsen, '34).

PLAN OF STUDY

Investigations of the protein minima for nitrogen equilibrium were attempted, using in all cases artificial 'synthetic' diets, wherein the protein was the only dietary variable operating. In his review of this subject, Cathcart ('21) states: "that the search for an absolute minimum is like the search of the philosopher for absolute truth. There is not one minimum but many protein minima—the minimum is a resultant of many factors." These numerous factors may be listed as follows: a) the nature of the foodstuffs fed with the protein; b) the completeness of the diet, both qualitatively and quantitatively; c) the caloric value of the food given; d) the degree of maturity of the experimental animal; e) the activity of the experimental animal; f) the environmental temperature; g) the nutritive condition of the animal, and h) an adequate preliminary adjustment period. In the present study attempts have been made to control the above factors.

The dogs were fed 'synthetic' diets: the chemical nature and exact proportions of all the nutrients were known. The plan of the diet followed the kilo-unit scheme, as suggested by Cowgill ('23). The components of the ration were the protein under investigation, hydrogenated vegetable oil,[2]

[2] 'Crisco,' obtained from the Proctor and Gamble Co., N. Y.

sucrose, dextrin, bone ash, modified Osborne and Mendel salt mixture (Wesson, '32) and the vitamin adjuvants. This combination was used during the study on serum protein but was later modified in that the fat component was replaced by lard and the dextrin by sucrose. These modifications seemed to render the diet more appetizing to our dogs subjected to plasmapheresis, which procedure proved to affect adversely the urge to eat. The determination of the minimal amount of protein in the diet to maintain nitrogen equilibrium required from three to six experimental rations for each of the proteins tested. To arrive at this value it was necessary to begin with a distinctly negative balance and by repeated protein increments attempt to attain equilibrium. Such a procedure is essential since nitrogen equilibrium may occur at different levels in a normal animal provided the protein intake is above the amount minimal for nitrogen equilibrium (Voit, 1881). In all cases in the present study the protein was increased gradually at the expense of an isodynamic portion of the carbohydrate fraction, the other constituents of the diet remaining constant. Thus, the nature of the foodstuffs fed with the protein was the same throughout the investigations. A variation of this factor may well produce fallacious results in that another variable is then introduced, namely, the protein-sparing action of carbohydrates versus that of fat (Lusk, '28; Maignon, '33). A representative series of diets is given in table 1 to illustrate how the dietary factors were controlled in the present investigation.

In our study it was desirable that the vitamin adjuvants should not introduce any appreciable amount of protein. As sources of vitamins A and D, two tablets of a cod liver oil concentrate[3] were given daily per 5 to 7 kg. of body weight. These tablets contain no protein. For vitamin B(B$_1$) an extract of rice polishing[4] was administered. The sample contained 56 international units per gram and was given

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[3] "White's Cod Liver Oil Concentrate Tablets," kindly furnished by the Health Products Corp., Newark, N. J.

[4] "Ryzamin," obtained from the Burroughs Wellcome Co., Tuckahoe, N. Y.

daily to the extent of 56 mg. per kilogram of body weight. This dosage of the vitamin is adequate and, although the extract contained 0.94% nitrogen, it introduced no appreciable source of dietary protein. As a source of vitamin $G(B_2)$ liver extract

TABLE 1

Casein diets constructed on the kilo-unit plan and employed in the determination of the minimal amount of this protein for nitrogen equilibrium

FOODSTUFF	GRAMS	CALORIES ABSOLUTE	CALORIES	COMPOSITION	CALORIES PER GRAM OF DIET	NITROGEN CONTENT
			%	%		%
Casein [1]	1.36	4.55	5.7	8.41		
	(1.14)			(7.07)		
Lard	3.77	34.00	42.5	23.27	4.94	1.122
Sucrose	10.36	41.45	51.8	64.00		
Bone ash	0.40	....	....	2.47		
Salt mixture	0.30	....	....	1.85		
Total	16.19	80.00	100.0	100.00		
Casein	1.85	6.25	7.8	11.38		
	(1.56)			(9.58)		
Lard	3.77	34.00	42.5	23.21	4.92	1.521
Sucrose	9.94	39.75	49.7	61.10		
Bone ash	0.40	....	....	2.46		
Salt mixture	0.30	....	....	1.85		
Total	16.26	80.00	100.0	100.00		
Casein	2.34	7.9	9.85	14.35		
	(1.97)			(12.07)		
Lard	3.77	34.0	42.50	23.08	4.89	1.918
Sucrose	9.53	38.1	47.65	58.28		
Bone ash	0.40	....	....	2.44		
Salt mixture	0.30	....	....	1.85		
Total	16.34	80.0	100.00	100.00		
Casein	2.52	8.5	10.6	15.40		
	(2.12)			(12.96)		
Lard	3.77	34.0	42.5	23.02	4.88	2.058
Sucrose	9.38	37.5	46.9	57.31		
Bone ash	0.40	....	....	2.44		
Salt mixture	0.30	....	....	1.83		
Total	16.37	80.0	100.0	100.00		

[1] 13.37% nitrogen; 84.1% pure.

The numbers in parentheses are the absolute values.

The vitamin adjuvants are described in the text.

no. 343[5] was given daily in the dosage of 210 mg. per kilogram of body weight. Our sample of liver extract no. 343 contained 8.1% of nitrogen of which we estimate 45% may be regarded as potentially protein nitrogen as a result of the analytic data obtained by Kapeller-Adler and Luisada ('34). Thus, the source of vitamin $G(B_2)$ yielded an appreciable amount of 'protein,' namely, 50 mg. per kilogram of body weight. At all the feedings the vitamin adjuvants[6] were administered apart from the food mixture, thus insuring an adequate vitamin intake by the animals.

The kilo-unit, as first described by Cowgill ('23) is designed to yield 80 calories. However, inasmuch as our dogs were kept in metabolism cages and their activity thus reduced, the daily administration of 70 calories per kilogram of body weight was found to be optimal for maintenance. The vitamin adjuvants yielded 1.5 calories per kilogram. With such a caloric intake the dogs maintained optimal and constant weights and always possessed hearty appetites at each feeding. The dogs were fed once each day, at as nearly the same hour as possible, this being between 9 and 10 A.M., and always consumed their ration within 5 to 10 minutes after it was offered. This feeding schedule was rigidly adhered to. Investigators (Krummacher, 1896; Gebhardt, 1897; v. Hoesslin and Lesser, '11) have repeatedly shown that if the food be distributed over the day and not concentrated in one meal, there tends to be a decrease in the amount of total nitrogen excreted. Chanutin and Mendel ('22), employing both low and high protein diets, were unable to observe any superior utilization of the dietary protein in dogs when subjected to fractional as compared with single feedings. Mitchell ('24 a) has confirmed these findings on rats fed a 5 to 10% protein diet, but reported a higher utilization of protein in the 10

[5] We are indebted to the Eli Lilly Co., Indianapolis, Ind., for a generous supply of this material in powdered form.

[6] It was found convenient to prepare an aqueous suspension of 75 gm. of liver extract no. 343 and 20 gm. of Ryzamin in 250 cc. of water. This suspension, adjusted to a pH just acid to· litmus, was given orally in the dosage of 0.7 cc. per kilogram of body weight.

to 18% diets when the daily rations were administered at regular intervals throughout the day. In order to avoid any question on this point, this variable was controlled by maintaining the feeding technic constant throughout the study. Furthermore, soon after the ingestion of a protein meal, there is a rapid rise in the output of nitrogen in the urine resulting ultimately in the excretion of 70% of the ingested protein within 8 hours (Veraguth, 1897; Gruber, '01; Haas, '08; Pepper and Austin, '15). Thus by feeding once a day at regular time intervals, there will be no tendency for 'carry-overs' in the nitrogen elimination from the previous periods.

The experimental animals were adult female dogs. Inasmuch as growing organisms require proportionally greater and variable nitrogen intakes, nitrogen balance studies are conducted more satisfactorily upon mature animals. The dogs were housed in metabolism cages where their activities were limited and maintained as constant as possible. The environmental temperature was poorly controlled, being dependent to a considerable extent upon the season of the year. Thus, the temperature of the dog room varied for the most part from 70 to 80°F. Moderate muscular exercise (Campbell and Webster, '21; Wilson, Long, Thompson and Thurlow, '25) and the temperature of the environment (Youngburg and Finch, '26) have never been demonstrated to exert any definite effect on nitrogen metabolism. However, the control of these two factors is essential in regulating the caloric balance which, in turn, may affect the nitrogen balance. Preliminary to the investigations on each of the proteins, the dogs were given a vermifuge and then given an excess of vitamin $B(B_1)$ to saturate the tissues with this dietary essential.[7] By preliminary feedings the weights of the animals were adjusted to an optimal nutritive condition, as estimated by the nutritive index formula (Cowgill, '28). The amounts of the diets fed, 70 calories per kilogram of body weight, were calculated on the basis of Cowgill's suggestion that a nutritive index value

[7] The source of the vitamin used here was 'Embo,' powdered wheat embryo, kindly furnished by the General Mills Inc., Minneapolis, Minn.

of 0.30 be regarded as the optimum. Throughout the experimental periods the greatest deviation of the dogs from this value was easily within ± 1.5%.

Preliminary to each of the collection periods, an adequate adjustment period is essential. Our most detailed study was conducted upon serum protein. Urinary nitrogen values were determined daily. Fecal nitrogens were determined over a 4-day period and averaged to give the daily aliquots. The values obtained indicate that a 5-day period is adequate under our experimental conditions for the organism to adjust itself to a new dietary regime. Subsequent studies on casein, lactalbumin, and gliadin were conducted with 5 days taken as the minimum for adjustment. The following 4-day urine samples were pooled and Kjeldahl determinations run in triplicate, the value obtained being translated to daily urinary nitrogens. A similar procedure was followed in determining the fecal nitrogens.

To insure as quantitative a collection of the excreta as possible, catheterizations were resorted to in the case of the urine samples, and charcoal was used to mark the feces. The urine was collected with toluene added as a preservative and sulfuric acid to prevent the volatilization of ammonia. Care was taken to wash the cages thoroughly each day, the washings being added to the urine. The feces were collected over the 4-day period and were preserved under acidified (sulfuric acid) alcohol. These were subsequently dehydrated on the steam bath by repeated evaporations of added alcohol. The dried product was ground, passed through a sieve to remove hair, and then analyzed for total nitrogen.

RESULTS

The serum protein minimum for nitrogen equilibrium

Beef serum was employed as the source of serum protein.

The blood was collected in oiled centrifuge bottles (500 cc.) and permitted to remain in the cold for 4 to 24 hours. The serum was removed by suction, the syneresis being hastened by centrifuging. The serum was then adjusted to pH 4.7

and added slowly to an equal volume of a boiling 20% NaCl solution, subjected to mechanical stirring. The coagulum was filtered, washed with hot water, and finally defatted and dehydrated by successive acetone and ether washings. The final product was air dried as 75 to 80°C. From 12 liters of blood 300 to 350 gm. of this crude serum protein could be obtained.

The nitrogen content of the preparation was 12.2% indicating 76.4% purity (Jones, '31). Moisture analyses of 16.00%, and sodium chloride determinations of 7.63% (Van Slyke and Sendroy, '23), accounted for the remaining 23.6% of impurity. No iron (Fe^{+++}) was present.

Six experimental periods, four on one dog and two on the other, were required to determine the minimal amount of serum protein in the diet capable of maintaining these dogs in nitrogen equilibrium. Four diets were employed. Adjusted to a nutritive index of 0.30, dog no. 8, a fox terrier, weighed 6.75 kg.; dog no. 1, a wire-haired terrier, 6.83 kg. In table 2 the results of these studies made with serum protein are summarized.

In the last column of table 2 are listed the data for several negative and two positive nitrogen balances. These values, expressed in terms of grams of nitrogen balance per kilogram of body weight at a nutritive index of 0.30, are plotted as ordinates in figure 1 against the per cent of calories furnished by the protein. By the method of least squares the average line, AB, is obtained giving equal weight to all points on the graph, i.e., of dogs no. 1 and no. 8. From the graph it is evident that the minimal amount of serum protein in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 8.5% of the caloric intake.

The casein minimum for nitrogen equilibrium

The sample of casein employed in this study was a commercial preparation,[8] which contained 13.37% nitrogen. This was indicative of an 84.1% purity (Jones, '31).

[8] Obtained from the Lister Bros., New York, N. Y.

TABLE 2

Determination of the serum protein minimum for nitrogen equilibrium

DOG NO.	EXPERI-MENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	*%*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
	3	5.7	86.5	426	2.5	10	0.970	0.111	....	....	6.91	Adjustment
	1	5.7	86.5	426	2.5	10	0.970	0.111	1.290	0.162	6.80	—0.371 gm. N
	4	7.0	97	473	2.5	10	1.320	0.111	....	....	6.77	Adjustment
	4	7.0	97	473	2.5	10	1.320	0.111	1.430	0.180	6.70	—0.179 gm. N
8	3	7.45	97	473	2.5	10	1.400	0.111	....	....	6.70	Adjustment
	4	7.45	97	473	2.5	10	1.400	0.111	1.460	0.181	6.66	—0.130 gm. N
	5	8.65	97	473	2.5	10	1.625	0.111	....	....	6.66	Adjustment
	2	8.65	97	473	2.5	10	1.625	0.111	1.538	0.189	6.61	+0.009 gm. N
	5	7.45	98	478	2.5	10	1.415	0.111	....	....	6.71	Adjustment
1	3	7.45	98	478	2.5	10	1.415	0.111	1.381	0.264	6.68	—0.119 gm. N
	5	8.65	98	478	2.5	10	1.640	0.111	....	....	6.72	Adjustment
	4	8.65	98	478	2.5	10	1.640	0.111	1.474	0.247	6.59	+0.030 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

Four experimental diets were employed in this study on four dogs. Dogs no. 1 and no. 8, used in the determination of the serum protein minimum, were no longer available. At a nutritive index of 0.30, dog no. 9, a spitz, weighed 8.00 kg.; dog no. 10, a fox terrier, 8.95 kg.; dog no. 11, a bulldog, 7.76 kg.;
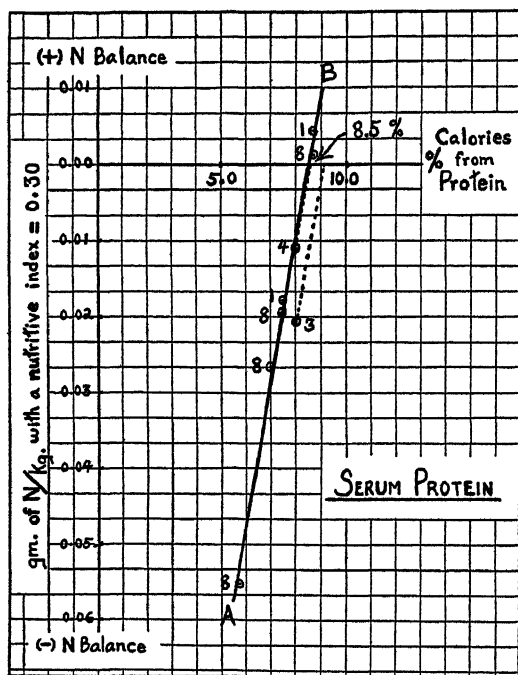
Fig. 1 The effect of increasing amounts of serum protein in the diet on the improvement in the negative nitrogen balance. The symbols, 1 and 8, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols, 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

and dog no. 12, a mongrel, 5.81 kg. Dog no. 12 was later found to be a coprophagist, and therefore no feces were obtained for analyses. This animal ate the diet poorly, about one-fourth of the ration being fed forcibly daily; it also showed a steady loss of weight. The dog was subsequently discarded. In table 3, summarizing the balance studies, are included the

TABLE 3

Determination of the casein minimum for nitrogen equilibrium

DOG NO.	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	*%*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
9	5	5.7	113.5	560	3.0	12	1.274	0.140	....	....	8.00	Adjustment
	4	5.7	113.5	560	3.0	12	1.274	0.140	1.695	0.233	8.00	−0.514 gm. N
	5	7.8	114	560	3.0	12	1.735	0.140	....	....	8.10	Adjustment
	4	7.8	114	560	3.0	12	1.735	0.140	1.813	0.250	8.04	−0.188 gm. N
	5	10.6	114.5	560	3.0	12	2.360	0.140	....	....	8.21	Adjustment
	4	10.6	114.5	560	3.0	12	2.360	0.140	1.960	0.258	8.17	+0.282 gm. N
10	5	7.8	127.5	627	3.25	13	1.940	0.158	....	....	8.87	Adjustment
	4	7.8	127.5	627	3.25	13	1.940	0.158	2.370	0.305	8.76	−0.577 gm. N
	5	10.6	128.5	627	3.25	13	2.642	0.158	....	....	8.95	Adjustment
	4	10.6	128.5	627	3.25	13	2.642	0.158	2.583	0.300	8.91	−0.083 gm. N
11	5	9.85	111	543	3.0	12	2.129	0.135	....	....	8.09	Adjustment
	4	9.85	111	543	3.0	12	2.129	0.135	1.980	0.276	8.06	+0.008 gm. N
12	7	7.8	83	407	2.5	10	1.262	0.100	....	....	5.30	Adjustment
	4	7.8	83	407	2.5	10	1.262	0.100	1.800	?	5.22	−0.438 gm. N
	6	10.6	83.5	407	2.5	10	1.720	0.100	....	....	5.12	Adjustment
	4	10.6	83.5	407	2.5	10	1.720	0.100	1.630	?	5.06	+0.190 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

values from this animal, using the urinary nitrogen data which were available.

In figure 2, these data are plotted according to the same scheme as that used for figure 1. The curve, AB, was calculated by the method of least squares, employing the results
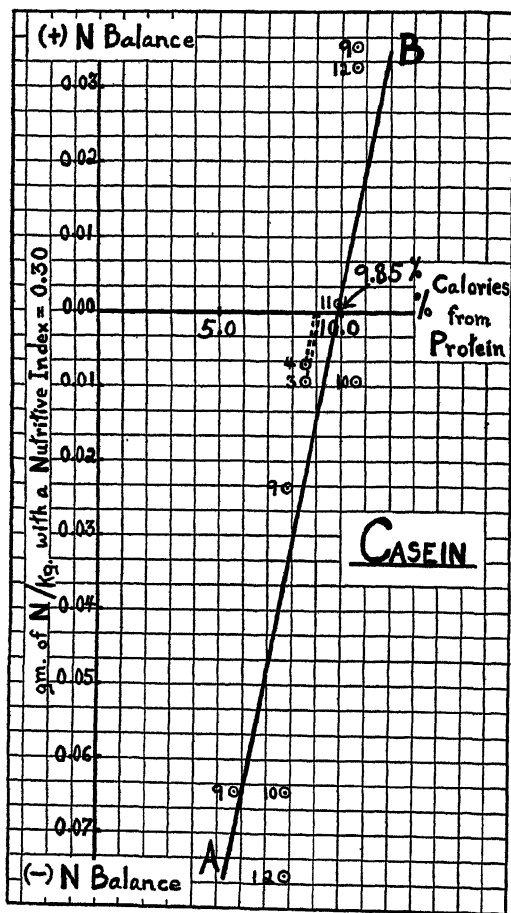
Fig. 2   The effect of increasing amounts of casein in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 10 and 11, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

obtained only from dogs no. 9, no. 10 and no. 11. From the figure it is evident that the minimal amount of casein in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 9.85% of the caloric intake.

The lactalbumin minimum for nitrogen equilibrium

The sample of lactalbumin employed in this study was a commercial preparation,[9] which contained 11.68% nitrogen. This was indicative of a 76.0% purity (Jones, '31).

Three experimental diets were employed in this study on three dogs. At a nutritive index of 0.30, dog no. 13, a mongrel, weighed 9.23 kg.; dog no. 9, 8.00 kg.; and dog no. 11, 7.76 kg. Table 4 summarizes the nitrogen balance studies made with lactalbumin as the protein.

In figure 3 the results are plotted, and a calculated curve, AB, drawn using the same scheme as that followed with the other proteins. From the figure, it is evident that the minimal amount of lactalbumin in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 6.75% of the caloric intake.

The gliadin minimum for nitrogen equilibrium

The gliadin employed in this study was obtained from gluten flour.[10] The method used in preparing the gliadin is that employed by Mr. L. S. Nolan of the Connecticut Agricultural Experiment Station. It gives large yields in relatively short periods when compared with the older methods. This procedure is described in detail in a communication from the experiment station (Nolan and Vickery, '36).

The nitrogen content of the preparation was 14.81%, indicating an 84.1% purity (Jones, '31). Gliadin prepared by the method mentioned above has been used in this laboratory in feeding experiments and has proven to be effective in stunting rats.

[9] Obtained from the National Milk Sugar Co., Bainbridge, N. Y.

[10] Obtained from the Battle Creek Food Co., Battle Creek, Mich. It is reported as 'containing 85% gluten.'

TABLE 4

Determination of the lactalbumin minimum for nitrogen equilibrium

DOG NO.	EXPERI-MENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	*%*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
13	5	5.7	131.5	645	3.5	14	1.410	0.162	....	....	9.47	Adjustment
	4	5.7	131.5	645	3.5	14	1.410	0.162	1.510	0.335	9.51	−0.273 gm. N
	5	6.5	132	645	3.5	14	1.608	0.162	....	....	9.53	Adjustment
	4	6.5	132	645	3.5	14	1.608	0.162	1.450	0.370	9.55	−0.050 gm. N
	7	7.45	132.5	645	3.5	14	1.840	0.162	....	....	9.46	Adjustment
	4	7.45	132.5	645	3.5	14	1.840	0.162	1.518	0.370	9.56	+0.114 gm. N
9	5	6.5	114.5	560	3.0	12	1.394	0.140	....	....	7.94	Adjustment
	4	6.5	114.5	560	3.0	12	1.394	0.140	1.243	0.231	7.98	+0.060 gm. N
	6	7.45	115	560	3.0	12	1.594	0.140	....	....	7.95	Adjustment
	4	7.45	115	560	3.0	12	1.594	0.140	1.305	0.240	7.94	+0.189 gm. N
11	6	7.45	112	543	3.0	12	1.560	0.135	....	....	8.02	Adjustment
	4	7.45	112	543	3.0	12	1.560	0.135	1.485	0.237	8.06	−0.027 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

It was noted that the dogs had some difficulty consuming the diet containing this protein due to the tendency for the gliadin to 'gum up' and stick to the teeth. The addition of an equal part of water to the diet produced a loose pasty mass which all the animals ate eagerly.
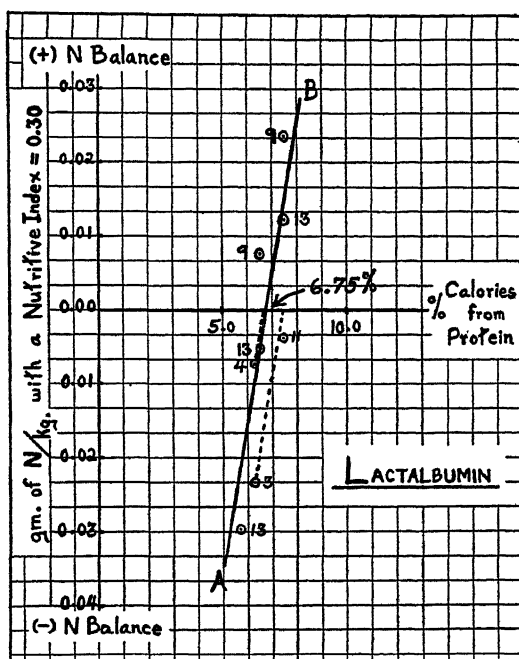
Fig. 3 The effect of increasing amounts of lactalbumin in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 11 and 13, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

When gliadin is fed as the sole protein, there is a deficiency with respect to the essential amino acid, lysine. Therefore, the length of the primary adjustment period was extended to 8 days. Subsequent adjustment periods were also greater than the usual 5 days as employed in the experiments with the other proteins. It is interesting to note here that Abder-

TABLE 5

Determination of the gliadin minimum for nitrogen equilibrium

DOG NO.	EXPERI-MENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
10	8	7.45	127.5	627	3.25	13	2.060	0.158	....	....	8.96	Adjustment
	4	7.45	127.5	627	3.25	13	2.060	0.158	2.470	0.229	8.84	−0.481 gm. N
	6	10.6	128.5	627	3.25	13	2.930	0.158	....	....	8.90	Adjustment
	4	10.6	128.5	627	3.25	13	2.930	0.158	3.266	0.198	8.92	−0.376 gm. N
	6	22.0	131.5	627	3.25	13	6.068	0.158	....	....	8.91	Adjustment
	4	22.0	131.5	627	3.25	13	6.068	0.158	6.030	0.239	8.90	−0.043 gm. N
11	8	10.6	111	543	3.0	12	2.530	0.135	....	....	8.09	Adjustment
	4	10.6	111	543	3.0	12	2.530	0.135	2.778	0.186	8.11	−0.299 gm. N
	6	16.0	112.5	543	3.0	12	3.830	0.135	....	....	8.10	Adjustment
	4	16.0	112.5	543	3.0	12	3.830	0.135	3.950	0.190	8.10	−0.175 gm. N
	8	25.0	115	543	3.0	12	5.992	0.135	....	....	8.09	Adjustment
	4	25.0	115	543	3.0	12	5.992	0.135	5.830	0.180	8.08	+0.117 gm. N
9	8	13.0	115	560	3.0	12	3.206	0.140	....	....	7.98	Adjustment
	4	13.0	115	560	3.0	12	3.206	0.140	3.375	0.201	8.00	−0.230 gm. N
13	8	13.0	132.5	645	3.5	14	3.699	0.162	....	....	9.46	Adjustment
	4	13.0	132.5	645	3.5	14	3.699	0.162	3.905	0.256	9.40	−0.300 gm. N
	8	25.0	136.5	645	3.5	14	7.112	0.162	....	....	9.52	Adjustment
	4	25.0	136.5	645	3.5	14	7.112	0.162	6.846	0.258	9.50	+0.170 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

halden ('12) in his studies with amino acid mixtures corresponding in composition to various native proteins experimented with mixtures simulating gliadin and found 3 to 5 days to be adequate for adjustment.

Nine experimental periods were employed in this study using the same dogs as in the other proteins studied. Six
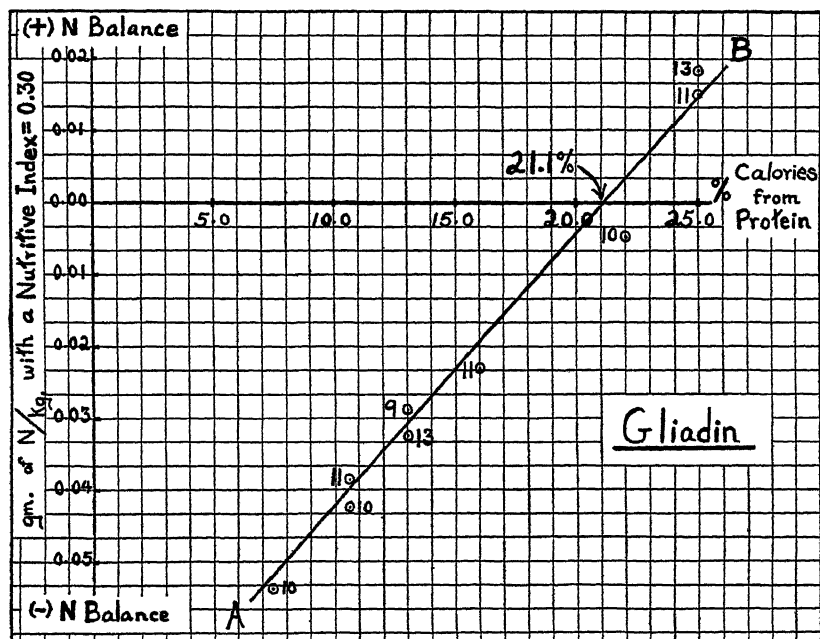
Fig. 4 The effect of increasing amounts of gliadin in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 10, 11 and 13, refer to the dogs yielding the data from which the calculated curve, AB, was drawn.

experimental diets were employed. The summary of these nitrogen balance studies is given in table 5.

Figure 4, which is similar in construction to the other figures, is based on the data of table 5. From the figure it is evident that the minimal amount of gliadin in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 21.1% of the caloric intake.

Peters and Van Slyke ('32) state that "any protein which is deficient in or entirely lacks one or more of these (essential) amino acids cannot be used alone to secure minimum nitrogen equilibrium. The amino acid deficiency becomes the limiting factor in the use of such a food" (p. 280).

Considering the results obtained in this study, it would seem that the lysine content of the gliadin is the limiting factor in the attainment of nitrogen equilibrium with this protein. Gliadin does contain 0.63% of this amino acid (Mendel, '23), but this evidently is not sufficient to permit normal growth as shown by the fact that young rats are stunted when fed gliadin diets. However, with respect to the attainment of nitrogen equilibrium in adult animals, it appears that sufficient lysine was ingested when the dogs consumed the high-gliadin diet which was associated with the positive balances recorded in the last column of table 5.

It is interesting to note that the fecal nitrogen excretions on the gliadin diets were always distinctly lower than those recorded for the same dogs when fed the casein or lactalbumin diets.

General applications

From a consideration of the various figures presented above, where at most four dogs were employed to obtain a maximum of nine points on the figure, it is evident that one cannot term these protein minima values as that amount of protein required by *every* dog to attain nitrogen equilibrium. It is apparent that many more animals must be used with each of the experimental periods to establish what may be regarded as a truly normal value having general significance. Furthermore, the application of these values as the protein minima without experimental confirmation on other dogs, particularly with reference to the animals subjected to plasmapheresis, could be justifiably criticized. For example, as exemplified by the casein figure (fig. 2) the values for one dog (see no. 9) may be as low as 8.5% and for another dog (no. 10) as high as 11.5%. However, the former animal shows a correspondingly

TABLE 6

Estimation of the protein minima for nitrogen equilibrium with dogs subjected to only one nitrogen balance study

DOG NO.	PROTEIN	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
								Basal	Vitamins	Urine	Feces		
		days	*%*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
3	Serum protein	6	8.0	240	1169	6.25	25	3.720	0.296	....	....	16.91	Adjustment
		4	8.0	240	1169	6.25	25	3.720	0.296	3.837	0.521	16.90	−0.342 gm. N
	Lact-albumin	6	6.25	239	1169	6.25	25	2.804	0.296	....	....	16.95	Adjustment
		4	6.25	239	1169	6.25	25	2.804	0.296	2.928	0.560	16.94	−0.388 gm. N
	Casein	6	8.5	238	1169	6.25	25	3.946	0.296	....	....	17.01	Adjustment
		4	8.5	238	1169	6.25	25	3.946	0.296	3.808	0.588	17.10	−0.154 gm. N
4	Serum protein	6	8.0	249	1211	6.5	26	3.860	0.303	....	....	17.04	Adjustment
		4	8.0	249	1211	6.5	26	3.860	0.303	3.883	0.472	17.14	−0.192 gm. N
	Lact-albumin	6	6.25	248	1211	6.5	26	2.908	0.303	....	....	17.50	Adjustment
		4	6.25	248	1211	6.5	26	2.908	0.303	2.855	0.480	17.45	−0.124 gm. N
	Casein	6	8.5	247	1211	6.5	26	4.095	0.303	....	....	17.30	Adjustment
		4	8.5	247	1211	6.5	26	4.095	0.303	3.985	0.534	17.25	−0.121 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

better utilization of casein at all levels of feeding as compared with the other dog. This indicates that within certain limits two dogs may differ as to their protein minimum, but that they tend to show a parallel consistency in their reactions to the various levels of dietary protein intake. This is obvious when one notes that the slopes of the figures indicating the responses of the dogs to the various levels of protein intake are remarkably parallel to each other. Thus, if we feed any dog one of the above proteins at a level which would be productive of a slightly negative balance, mere extrapolation to the line of nitrogen equilibrium by drawing a line parallel to

TABLE 7

Protein minima for nitrogen equilibrium

DOG NO.	PROTEIN	CALORIES FROM PROTEIN
		%
3	Serum protein	9.1
	Lactalbumin	7.4
	Casein	9.1
4	Serum protein	8.5
	Lactalbumin	6.65
	Casein	8.9

the calculated curve makes it possible to estimate the protein minimum for that animal by means of only *one* nitrogen balance study. The fact that the balance should be slightly negative is desirable in that any differences in the slopes of the dog's responses to the dietary protein from that of the calculated curve will be reduced to a minimum.

This scheme has been applied in evaluating the protein minima for our dogs to be subjected to plasmapheresis. At a nutritive index of 0.30 dog no. 3, a female hound, weighed 16.70 kg.; dog no. 4, a female collie, 17.30 kg. The gliadin value was not determined inasmuch as possible toxic manifestations were feared (Melnick, '36). In table 6 is found a summary of these nitrogen balance studies.

These data, when treated according to the scheme followed in preparing the various figures, were plotted on the corresponding charts. By extrapolating to the line of nitrogen equilibrium in accordance with the plan stated above, the protein minima presented in table 7 were obtained.

DISCUSSION AND CONCLUSIONS

The casein value of 9.85%, obtained with dogs no. 9, no. 10 and no. 11 appears to be somewhat too high. The values obtained with the dogs no. 3 and no. 4, which were later subjected to plasmapheresis, have been averaged with those yielded by the other dogs, arbitrarily allotting double weight to the latter values because more points were obtained for those animals. These calculations yield the following as the protein minima for nitrogen equilibrium, expressed in terms of the per cent of total ingested calories furnished by the protein in diets such as are described in this paper:

Lactalbumin	Serum protein	Casein	Gliadin
6.9%	8.6%	9.4%	21.1%

According to these figures the relative biological values of these proteins for the attainment of nitrogen equilibrium may be listed as follows, assigning to lactalbumin the value of 100:

Lactalbumin	Serum protein	Casein	Gliadin
100	80	73	33

When comparing these values with those reported in the literature (Boas-Fixsen, '34), it is pertinent to point out that most of the biological values recorded by others were obtained from experiments made on another species, namely, the rat. Furthermore, the criteria employed in these other studies have not been the same. In some cases the proteins have been evaluated with respect to their ability to promote growth; in other experiments the amounts of the proteins needed for maintenance were determined. The influence of various proteins upon fertility, lactation, longevity and stability of the nervous system has also been studied. Nitrogen balance studies have also been conducted with the rat,

the biological value of the protein being based upon the percentage of absorbed nitrogen that escaped excretion through the kidney. In listing the above relative values for the proteins studied in the present investigation, we emphasize that they represent only the respective abilities of these foodstuffs to satisfy the general nitrogen requirements of the dog as evidenced by the attainment of nitrogen equilibrium. Furthermore, it should be clearly understood that these minimal values are not to be regarded as reproducible unless the same experimental technique, as outlined in the present paper, is followed. This point seems obvious, since the minimal amount of a protein needed for nitrogen equilibrium is not a fixed constant but a resultant of the operation of many factors, as described above in the section on 'Plan of study.' Thus, by merely changing the experimental conditions, it is quite possible that large variations in these protein minimal values would be obtained, even with the same dogs used as the experimental animals. Our data would seem to gain in significance because of the fact that for the most part, each comparative test of these proteins under our fixed conditions was made on the same animal, and the relative ratings yielded by the different animals were essentially the same.

SUMMARY

a. Lactalbumin, serum protein, casein and gliadin have been evaluated with respect to the minimal amount of each in the diet essential for the dog in attaining nitrogen equilibrium. Expressed as percentage of total ingested calories furnished by the protein, these values have been found to be 6.9, 8.6, 9.4 and 21.1%, respectively. The corresponding relative biological values for these proteins were listed as 100, 80, 73 and 33.

b. A method for the estimation of the protein minimum for nitrogen equilibrium for any dog, employing only one experimental period, has been described.

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THE VITAMIN B AND VITAMIN C CONTENT OF MARINE ALGAE.

EARL R. NORRIS, MARY K. SIMEON AND HAL B. WILLIAMS

Oceanographic Laboratories and Division of Biochemistry, University of Washington, Seattle

Marine algae are relatively simple, chlorophyllous plants which usually grow either under or partly under water. They are not differentiated into root, stem and leaf, and are reproduced by spores instead of seeds. The large marine algae are often given the common name of seaweeds, and are designated according to color as green, red and brown algae. Chlorophyl gives the characteristic green color to the green algae (Chlorophyceae). The green color of the chlorophyl is almost completely masked by a red pigment in the red algae (Rhodophyceae). A brown pigment is found in the brown algae (Phaeophyceae) in addition to the chlorophyl. The real basis for the division of the algae into these three subclasses is certain differences in their method of reproduction, but the colors correspond so closely with these differences in reproduction that it is usually possible to assign an alga to its proper subclass by its color.

Marine algae or seaweeds, together with phytoplankton, play the same role in the life and nutrition of the sea that land plants do in terrestrial life, and must therefore be expected to satisfy the nutritional requirements of much of marine life.

Although very little seaweed is eaten in America, in many countries, especially in the Orient, algae are consumed in large quantities. A summary of the utilization of edible algae is given in 'Marine Products of Commerce,' (Tressler,

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'23). Seaweeds contain no starch and the complex carbohydrates which are present are not readily hydrolyzed by the human digestive enzymes.

The value of marine algae as a food is due mainly to the inorganic salts present and the vitamins. Seaweeds have long been used for the prevention of goiter and for the prevention and cure of scurvy.

Very little work has been reported upon the vitamin content of marine algae. Two varieties of Hawaiian edible algae, limu eleele (a species of Enteromorpha) and limu lipoa have been tested (Miller, '27) and found to be poor sources of vitamins B and C.

A number of algae were collected in the region of the San Juan Archipelago during June, July and August of two successive years. Some of the species collected are used as food by Indians of the Pacific coast, and many of the species are of the same type and genus as those eaten in various parts of the world. Vitamin B and vitamin C were determined on some of the algae collected.

VITAMIN B

For the determination of vitamin B the algae were cleaned, air dried in the shade, and stored to be tested during the winter months.

Vitamin B(B_1) was determined by feeding tests (Chase and Sherman, '31). The rats which were used were weaned at 28 days, placed in individual cages with screen bottoms, and given a basal diet as follows, with distilled water for drinking:

The basal diet

	%
Casein (extracted)	18
Autoclaved yeast	10
Osborn and Mendel salt mixture	4
Cod liver oil	1
Olive oil	9
Starch	58

In extracting the casein, 400 gm. were stirred with 1.5 liters of 60% alcohol for 30 minutes, then allowed to stand over night. The alcohol was removed in a hydraulic press at 5000

pounds per square inch. The extraction with 60% alcohol was repeated, and the casein stirred with 95% alcohol for 1 hour, pressed, and dried at room temperature. The autoclaved yeast was heated at 20 pounds pressure for 4 hours, and then tested for the absence of the heat labile factor measured as $B(B_1)$ and the presence of the heat stable factor measured as $G(B_2)$. The Osborn and Mendel salt mixture was modified by the addition of 0.161 gm. of hydrated copper sulfate for every 134.8 gm. of calcium carbonate used in the mixture. Negative controls were run, feeding only the basal diet and distilled water, on which growth ceased uniformly near the end of the second week. Positive controls were run

TABLE 1

The average weekly gain in weight, in grams, of experimental animals fed graded amounts of algae during a 4-week experimental period

GRAMS OF ALGAE FED PER RAT PER DAY	ALARIA VALIDA	ENTEROMORPHA INTESTINALIS	LAMINARIA BULLATA	PORPHYRA NEREOCYSTIS	PORPHYRA PERFORATA	RHODYMENIA PERTUSA	ULVA LACTUCA
0.1	....	....	....	—2.4	—1.6	....	—2.4
0.2	—1.6	—6.5	....	5.7	1.6	—1.7	....
0.3	—0.3	—3.5	—1.7	8.7	6.3	1.3	3.5
0.5	6.2	—1.6	3.1	....	17.3	4.3	12.0
0.6	....	....	6.0	....	....	....	....
0.8	11.5	....	....	....	....	10.0	....

by supplementing the basal diet with an alcoholic extract of wheat germ which had been shown to contain the heat labile factor and be free of the heat stable factor, normal growth was obtained in every case.

To determine the vitamin $B(B_1)$ in marine algae, the animals were first depleted of vitamin B by feeding the basal diet until cessation of growth and the animals started to lose weight, which required about 2 weeks. The diet was then supplemented by graded amounts of the substance to be tested. The algae were ground and fed separately in weighed portions, which were consumed quantitatively. Both sexes of rats were used, and the litter mates and sexes distributed among the groups on different tests to give reliable results.

The results obtained are given in table 1.

The unit of vitamin B for each seaweed was calculated using the Sherman unit (Sherman, '32) which is defined as: "a unit of vitamin B may be taken as that amount which when fed daily under experimental conditions as described will induce in a standard test animal an average gain of 3 gm. per week during the test period." Table 2 gives the values obtained. The results on a good grade brewers yeast fed under the same conditions is given for comparison.

TABLE 2

The vitamin B content of marine algae

MATERIAL TESTED	WHERE OBTAINED	GRAMS FED PER DAY FOR A GAIN IN WEIGHT OF 3 GM. PER WEEK	UNITS (SHERMAN) OF VITAMIN B (B_1) PER GRAM
Phaeophyceae:			
Alaria valida	Upper sub-littoral zone	0.4	2.5
Laminaria sp.	Dredged from a depth of 5 to 10 fathoms	0.5	2.0
Rhodophyceae:			
Porphyra nereocystis	Surface	0.18	5.5
Porphyra perforata	Littoral zone	0.2	5.0
Rhodymenia pertusa	Dredged from a depth of 5 to 10 fathoms	0.45	2.2
Chlorophyceae:			
Enteromorpha sp.	Littoral zone	. . . .	Trace only
Ulva lactuca	Surface	0.25	4.0
Dried brewers yeast	. . . . . .	0.075	13.3

From the data in table 2 it is shown that of the algae tested several are very good sources of vitamin B, comparing favorably with many common fruits and vegetables. Porphyra was found to be the best source of the algae tested and Enteromorpha the poorest source. Enteromorpha had only a trace of vitamin B present. No relation was found between the source of the algae, that is the depth from which it was obtained, and the vitamin B content.

VITAMIN C

Much of the seaweed collected for shipment to the market is dried and prepared in such a manner as to destroy any

vitamin C which might have been present; however, among peoples living on the coast fresh seaweeds have been an important part of the diet.

Vitamin C (ascorbic acid) was determined in the fresh algae, by titration with 2-6-dichlorophenolindophenol, using the method of Bessey and King ('33). The algae were blotted free of excess water with a clean towel or filter paper immediately before analysis.

Before applying a titration method such as that of Bessey and King to a new type of food product, the chemistry of which is as little known as the chemistry of the algae, it is first necessary to test the method to be sure that the reducing substance being measured is ascorbic acid. The validity of the determination was tested by two methods. The first method was the precipitation of interfering substances with mercuric acetate by the procedure of Emmerie and Van Eekelen ('34), which includes the use of hydrogen sulfide and would therefore insure the reduction of any oxidized ascorbic acid, if it were present in the oxidized form. The second was the determination of vitamin C by the method of Tauber and Kleiner ('35), using a specific oxidase for ascorbic acid.

The ascorbic acid was determined upon extracts of Porphyra perforata and Ulva lactuca, using the method of Emmerie and Van Eekelen ('34). The extract of the seaweed was treated with a solution of mercuric acetate to precipitate substances other than ascorbic acid which might reduce the dye used for titration. The precipitate was removed by centrifuging, and the supernatent liquid treated with hydrogen sulfide to remove the excess of mercury. The hydrogen sulfide was removed with nitrogen. Titrations were made on the original extract and the solution after precipitation. The results showed that 99% of the reducing power measured as vitamin C in Porphyra remained after precipitation and treatment with hydrogen sulfide, and 95% of the reducing power remained in the extract of Ulva. A sample of Rhodymenia which gave a titration indicating less than 0.01 mg. of vitamin C per gram gave the same results after treatment, indicating that the ascorbic acid was not present in the oxidized form.

Extracts of Porphyra perforata and Ulva lactuca prepared according to the method of Bessey and King ('33) and an extract of Porphyra treated with mercuric acetate and hydrogen sulfide according to the method of Emmerie and Van Eekelen ('34) were oxidized with a specific oxidase prepared according to the method of Tauber and Kleiner ('35), who state that the reducing substance removed may be considered as 'true' ascorbic acid.

Enzyme solutions were prepared by the extraction of both yellow crookneck squash, and summer squash. Both enzyme preparations gave 100% oxidation of the ascorbic acid of lemon juice in 30 minutes. The enzyme oxidized from 96% to 100% of the reducing power of Porphyra and 73% of the reducing power of Ulva. Controls were run using boiled enzyme preparation, and incubation without enzyme to check on the possibility of auto-oxidation. The results indicate that the reducing substance in algae measured by titration with 2-6-dichlorophenolindophenol is ascorbic acid.

Using the method of Bessey and King titrations were made upon a number of marine algae; the results given in table 3 are calculated to the blotted wet weight. Several samples of each species were analyzed and the average of the results reported.

Results obtained by titration of twenty-four samples of lemons obtained from the local market during the same period gave an average value of 0.43 mg. of vitamin C per gram of lemon juice, varying from 0.31 to 0.57 which is appreciably lower than the average value of 0.57 for lemon juice given by Bessey and King ('33). From the table it will be seen that some of the seaweeds are as high or even higher in vitamin C than lemons purchased on the local market at the same time of year.

While no hard and fast rule can be drawn, in general those forms collected from the littoral and upper sublittoral zones were higher in vitamin C than forms dredged from considerable depth. Algae dredged from depths of 5 to 10 fathoms were practically devoid of ascorbic acid. Two samples of

Rhodymenia which had no vitamin C when dredged were stored in a live box at the surface for several days and upon titration gave values of 0.07 and 0.08 mg. per gram of algae indicating that under certain conditions probably controlled principally by light Rhodymenia was able to form and store vitamin C.

TABLE 3

Name of algae	Vitamin C milligrams per gram wet weight	
	1934	1935
Phaeophyceae (brown algae):		
From the surface, littoral and upper sublittoral zones:		
Alaria valida	0.53	
Egregia menziesii	0.04	
Fucus evanescens	0.24	0.36
Hedophyllum sessile	0.21	
Macrocystis pyrifera	0.19	
Postelsia palmaeformis	0.09	
Dredged from a depth of 5 to 10 fathoms:		
Agarum fimbriatum	0.02	
Costaria costata	0.02	
Desmarestia munda	0.01	0.01
Laminaria bullata	0.02	0.01
Chlorophyceae (green algae):		
From the surface and littoral zone:		
Enteromorpha sp.	0.15	0.11
Ulva lactuca	0.46	0.38
Rhodophyceae (red algae):		
From the surface, littoral and upper sub-littoral zones.		
Gigartina papillata	0.41	0.25
Grateloupia Cutleriae	Less than 0.01	
Halosaccion glandiforme	0.13	
Iridaea sp.	0.26	
Porphyra naiadum	0.36	
Porphyra nereocystis	0.53	
Porphyra perforata	0.60	
Prionitis Lyallii	0.03	
Turnerella pacifica		0.09
Dredged from a depth of 5 to 10 fathoms:		
Agardhiella tenera	Less than 0.01	
Anatheca furcata	Less than 0.01	
Callophyllis sp.		Less than 0.01
Dasyopsis plumosa	Less than 0.01	
Hymenena sp.	Less than 0.01	
Opuntiella californica	Less than 0.01	
Polyneura latissima	Less than 0.01	Less than 0.01
Rhodymenia pertusa	Less than 0.01	Less than 0.01

The local species of Porphyra were found to be very good sources of vitamin C; they are used as food by the Indians of the region and of Alaska. Various other species of Porphyra are widely used in different parts of the world (Tressler, '23) under the names of red laver in England, sloke in Ireland, slack in Scotland and amanori in Japan, where large quantities of it are cultivated and some of the crop eaten fresh. Ulva sp., called green laver, oyster green and sea lettuce, is also a good source of ascorbic acid. Alaria sp. or murlins is gathered and eaten on the coast of Scotland, Ireland and Iceland; the local species proved to be a good source of vitamin C. While other varieties of algae such as Rhodymenia sp., known as dulse, dilling, dulling and water leaf, together with many of the deep-sea red algae: as, Agardhiella, Polyneura, Dasyopsis, etc., were very poor sources of ascorbic acid.

CONCLUSIONS

1. Six of the seven algae tested were found to be good sources of vitamin B, comparing favorably with many fruits and vegetables.

2. Species of Porphyra were found to be richest source of both vitamin B and vitamin C among the algae tested.

3. A few of the species of algae tested were found to be as rich a source of vitamin C as lemons bought on the local market.

4. Algae growing in the littoral zone or on the surface tend to be higher in vitamin C than algae which are dredged from a depth of 5 to 10 fathoms.

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THE MULTIPLE NATURE OF THE VITAMIN D OF FISH OILS [1]

CHARLES E. BILLS, O. N. MASSENGALE, MIRIAM IMBODEN AND
HELEN HALL

Research Laboratory, Mead Johnson and Company, Evansville, Indiana

In the introductory paper of this series (Bills, '35) the available evidence on the multiple nature of vitamin D was brought forth, and the conclusion reached that vitamin D exists in at least six chemically distinct forms. A seventh and an eighth form were subsequently recognized (Bills, '36). It was shown that none of these has been identified with the vitamin D of fish oils, and that the latter may be additional to the forms artificially prepared.

Until recently it was generally assumed that the vitamin D of fish oils is a single substance. Its concentration was known to vary widely in the liver oils of different species, or even of a particular species, but that the vitamin varies in kind, as well as in amount, was not appreciated before the investigation of tuna liver oil by Bills, Massengale and Imboden ('34). In that work we showed that the liver oil of one of the tuna species was less antirachitic than cod liver oil, rat unit for rat unit, in chickens. In this respect it differed from halibut liver oil, which resembled cod liver oil to within the errors of assay.

That certain fish oils have approximately the same chicken effectiveness, per rat unit, as cod liver oil is not remarkable. It is, however, a coincidence that several of these should be among the first that were studied. Thus sardine body oil,

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which Bills ('27) found equal to cod liver oil in assays with rats, has for a number of years been replacing cod liver oil in poultry raising. Rygh ('35) was unable to recognize any difference in chicken effectiveness, per rat unit, in the vitamin D of seventeen species of fish. Haman and Steenbock ('36) found that sardine oil, as well as halibut liver oil and the liver oil of burbot (a fresh water cod), had about the same efficacy as cod liver oil, per rat unit, for chickens. Black and Sassaman ('36) found that the liver oils of cod, halibut, swordfish and Japanese mackerel were about equally effective on chickens, per rat unit.

In the work of Rygh ('35) and especially of Dols ('35, '36) the vitamin D of tuna liver oil was found to be as effective as that of cod liver oil, per rat unit, for chickens. It will be shown, however, that these workers dealt with a species of tuna different from the one used by us. Haman and Steenbock ('36) observed that a commercial, mixed species, tuna liver oil was 'somewhat less effective' on chickens, per rat unit, than the other oils which they studied. Black and Sassaman ('36) in repeated trials with three Japanese species of tuna (bluefin, striped and yellowfin) found that the liver oils were only from 40 to 63% as effective, rat unit for rat unit, as cod liver oil on chickens.

The present report gives the details of our original experiments with the liver oils of halibut and California bluefin tuna, together with studies made with the oils of numerous other species of fish.

PROCEDURE

The means for distinguishing the forms of the vitamin was the differential response of rats and chickens. The oils, suitably diluted in maize oil, were assayed with rats and again with chickens, and the relative effectiveness of the vitamin D for chickens, rat unit for rat unit, was expressed on the basis of cod liver oil = 100. This was the device employed by Massengale and Nussmeier ('30) and by other workers in distinguishing the vitamin D of irradiated ergosterol from that of cod liver oil.

The assays with rats were conducted essentially in accordance with the technic of Bills, Honeywell, Wirick and Nussmeier ('31). This was modified to the extent of including the use of a reference oil (a replica of the international standard) in parallel with the test oil, and of a correspondingly revised procedure for the calculation of probable error. In every assay at least twenty litter-mate pairs of rats were used in the final assembly, in addition to those used in exploratory assays above and below the critical level. Thus we obtained assays of unusual accuracy, the probable errors of which ranged from ±5% to ±7%, inclusive of the probable error of the reference replica. The potencies were expressed in international units (I.U.) of vitamin D per gram of oil. Needless to say, the oils which had been assayed with rats were kept under conditions which permitted no measurable loss of potency before they were assayed with chickens. In some instances, as a double check, the oils were re-assayed with rats after the assays with chickens were completed.

The assays with chickens were conducted according to the quantitative method of Massengale and Bills ('36). It should be pointed out that this new method was designed particularly for the detection of small differences in antirachitic potency with the chicken, and that it is the only method employing this species which includes means of calculating probable error. At least ten chicks were used in the assay of each oil. The probable errors ranged from ±8% to ±13% of the number of I.U. of vitamin D found.

The vitamin A determinations were made photometrically, with the apparatus known as the Hilger Vitameter. After suitable corrections for irrelevant absorption, they are probably at least as accurate as bioassays for this vitamin.

The oils studied are described below. All but nos. 27, 29, 30 and 31 were rendered either in this laboratory or in the oil factory of our company. With these exceptions, all livers were shipped from the place of origin in the frozen state. The identity of each kind was confirmed by inspection. In the following list, care was taken to use the zoölogical names

favored by the leading authorities, but the present state of fish taxonomy is such that the choice was not in every case wholly satisfactory.

Order Heterosomata (flatfishes)

Oil no. 1. Halibut. Hippoglossus hippoglossus (H. stenolepis). The fish were caught in the Pacific Ocean between northern California and Unalaska. The oil represented 26,000 kg. of livers. No adulteration. Potency, 41,000 I.U. of vitamin A and 1400 I.U. of vitamin D (rat) per gram.

Oil no. 2. Round-nosed sole. California sole. Eopsetta jordani. The fish were caught in Puget Sound, near Seattle. One hundred and eighty-one kilograms of livers. No adulteration. Potency, 110,000 I.U. of vitamin A and 1100 I.U. of vitamin D (rat) per gram.

Order Percomorphi, series Scombriformes
(scombroid fishes: the tunas)

Oil no. 3. Bluefin tuna (of California). Leaping tuna, Thunnus saliens. Formerly and still most commonly, but wrongly, designated T. thynnus. Pacific Ocean, off southern California. Laboratory sample of 2 kg. of livers. No adulteration. Potency, 84,000 I.U. of vitamin A and 46,000 I.U. of vitamin D (rat) per gram.

Oil no. 3-x. Unsaponifiable fraction of oil no. 3, concentrated further by elimination of the cholesterol. Potency, 770,000 I.U. of vitamin A and 800,000 I.U. of vitamin D (rat) per gram (some vitamin A lost in saponification).

Oil no. 7-a. Bluefin tuna (of New England). Sometimes locally known as horse mackerel. Thunnus secundodorsalis. More commonly designated T. thynnus, but probably not identical with the latter. Provincetown, Massachusetts. The laboratory sample of 8 kg. of livers was kindly contributed by Mr. Harden F. Taylor of the Atlantic Coast Fisheries Co. No adulteration. Potency, 80,000 I.U. of vitamin A and 16,000 I.U. of vitamin D (rat) per gram.

Oil no. 9. Bluefin tuna (of Japan). Oriental tuna. In Japanese, 'maguro.' Thunnus orientalis. Formerly confused by some authors with T. thynnus. Pacific Ocean, off Japan. Four thousand one hundred and ninety-six kilograms of livers. Probably no adulteration. Potency, 36,000 I.U. of vitamin A and 61,000 I.U. of vitamin D (rat) per gram.

Oil no. 12. Albacore. Long-finned tuna. In Japanese, 'binnaga.' Thunnus germo (Germo germo). Pacific Ocean, off Japan. Two thousand seven hundred and seventy-six kilograms of livers. No adulteration. Potency, 18,000 I.U. of vitamin A and 41,000 I.U. of vitamin D (rat) per gram.

Oil no. 13. Yellowfin tuna. Yellowfin albacore. Neothunnus macropterus. Pacific Ocean, off Mexico and Central America. Nine thousand and eighty kilograms of livers. No adulteration. Potency, 48,000 I.U. of vitamin A and 13,000 I.U. of vitamin D (rat) per gram.

Oil no. 14. Striped tuna. Oceanic bonito. Commonly called skipjack in California. Katsuwonus pelamis. Pacific Ocean, off Mexico. Four hundred and fifteen kilograms of livers. No adulteration. Potency, 43,000 I.U. of vitamin A and 58,000 I.U. of vitamin D (rat) per gram.

Oil no. 15. California bonito. Sometimes called skipjack in California. Sarda lineolata. Pacific Ocean, off southern California and northern Mexico. One thousand three hundred and one kilograms of livers. No adulteration. Potency, 120,000 I.U. of vitamin A and 50,000 I.U. of vitamin D (rat) per gram.

Oil no. 16. California mackerel. Pacific mackerel. Pneumatophorus diego. Pacific Ocean, off southern California. Two thousand nine hundred and ninety-three kilograms of livers. Perhaps slightly adulterated with the California horse mackerel, Trachurus symmetricus. Potency, 88,000 I.U. of vitamin A and 1400 I.U. of vitamin D (rat) per gram.

Order Percomorphi, continued (miscellaneous percomorphs)

Oil no. 17. Swordfish. Xiphias gladius. Atlantic Ocean, from Massachusetts to Nova Scotia. Two thousand eight hundred and six kilograms of livers. No adulteration. Potency, 250,000 I.U. of vitamin A and 14,000 I.U. of vitamin D (rat) per gram.

Oil no. 18. Black sea-bass. California jewfish. Stereolepis gigas. Pacific Ocean, off northern Mexico. One thousand six hundred and two kilograms of livers. No adulteration. Potency, 520,000 I.U. of vitamin A and 4300 I.U. of vitamin D (rat) per gram.

Oil no. 19. Cabrilla. Epinephelus analogus. Gulf of California, northern part. Nine hundred and forty-six kilograms of livers. No adulteration. Potency, 170,000 I.U. of vitamin A and 220 I.U. of vitamin D (rat) per gram.

Oil no. 20-a. White sea-bass. Cynoscion nobilis (Atractoscion nobilis). Pacific Ocean, off southern California. One thousand five hundred and ninety-five kilograms of livers. No adulteration. Potency, 55,000 I.U. of vitamin A and 6000 I.U. of vitamin D (rat) per gram.

Oil no. 21. Totuava. Eriscion macdonaldi. Gulf of California, northern part. Two thousand eight hundred and eighty kilograms of livers. No adulteration. Potency, 56,000 I.U. of vitamin A and 1200 I.U. of vitamin D (rat) per gram.

Order Cataphracti (rockfishes, etc.)

Oil no. 22. Sablefish. 'Black cod' (not a codfish). Anoplopoma fimbria. Pacific Ocean, from nothern California to Alaska. Sixteen thousand and thirty-eight kilograms of livers. No adulteration. Potency, 82,000 I.U. of vitamin A and 340 I.U. of vitamin D (rat) per gram.

Oil no. 23-a. 'Lingcod' (not a codfish). Ophiodon elongatus. Pacific Ocean, from northern California to Alaska. Seven thousand five hundred and sixty-three kilograms of livers. No adulteration. Potency, 160,000 I.U. of vitamin A and 840 I.U. of vitamin D (rat) per gram.

Oil no. 24. Bocaccio. Sebastodes paucispinis. Pacific Ocean, off California. Laboratory sample of 7 kg. of livers. No adulteration. Potency, 77,000 I.U. of vitamin A and 2000 I.U. of vitamin D (rat) per gram.

Oil no. 25. Chili-pepper. Sebastodes goodei. Pacific Ocean, off California. Laboratory sample of 1 kg. of livers. No adulteration. Potency, 150,000 I.U. of vitamin A and 270 I.U. of vitamin D (rat) per gram.

Order Jugulares

Oil no. 26. Wolffish. Ocean catfish. Anarhichas lupus. Atlantic Ocean, off Nova Scotia. Laboratory sample of 6 kg. of livers. No adulteration. Potency, 1300 I.U. of vitamin A and 19 I.U. of vitamin D (rat) per gram.

Order Euselachii

Oil no. 27. Basking shark. Cetorhinus maximus. Pacific Ocean, off southern California. The oil was obtained from Mr. J. F. Grundell of Monterey, who rendered it from a few kilograms of livers. Probably no adulteration. Potency, no detectable amount of vitamin A; about 4 I.U. of vitamin D (rat) per gram.

Order Tectospondyli

Oil no. 28. Dogfish. Squalus suckleyi. Pacific Ocean, off California. Laboratory sample of 3 kg. of livers. No adulteration. Potency, 23,000 I.U. of vitamin A and 16 I.U. of vitamin D (rat) per gram.

Order Anacanthini, family Gadidae (codfishes)

Oil no. 29. Pollack. Pollachius virens. Atlantic Ocean, off Maine. About 25,000 kg. of livers. Adulteration probably trivial, if any. Potency, 2800 I.U. of vitamin A and 110 I.U. of vitamin D (rat) per gram.

Oil no. 30. Hake. Urophycis chuss and U. tenuis. (Very similar species, perhaps intergrading.) Atlantic Ocean, off Maine. About 50,000 kg. of livers. Adulteration probably trivial, if any. Potency, 2300 I.U. of vitamin A and 130 I.U. of vitamin D (rat) per gram.

Order Isospondyli

Oil no. 31. Sardine. Sardinia caerulea. Pacific Ocean, off southern California. Poultry oil, manufactured from the entire fish, and from trimmings and entrails, including liver. About 65,000 kg. of fish. Adulteration trivial, if any. Potency, 2700 I.U. of vitamin A and 110 I.U. of vitamin D (rat) per gram.

FINDINGS AND DISCUSSION

The oils in the above list represent twenty-five species. They include a number of the most important vitamin sources, as well as several of merely theoretical interest. The potencies are not necessarily typical of the species, even when the amount of livers represented is large. For example, 26 metric tons of halibut livers were taken for oil no. 1, but they were all from fish caught in the summer months, when the oil yield is high and the potency low. The cod liver oil, with which the master curve of response was originally established, was described in the paper on technique (Massengale and Bills, '36). In the present work, another cod liver oil of specially determined potency was administered from time to time, to make certain that the response of the chicks was normal.

The experimental findings are summarized in table 1. The table shows for each oil the number of I.U. of vitamin A and

TABLE 1
Relative effectiveness of vitamin D from different sources for rats and chickens

OIL NO.	NAME OF FISH (OR STEROL)	VITAMINS GIVEN PER 100 GM. DIET			RESPONSE OBTAINED			EFFICACY RATIO	
		A I.U.	D I.U.	D PE, %	Femur ash, %	I.U. of D/100 gm.	PE, %	C.L.O. = 100	PE, %
1	Halibut	510	17.5	±7	45.42	15.1	+9; −9	86	+11; −11
2	Round-nosed sole	1900	18.9	6	45.57	15.4	9; 9	81	11; 11
3	Tuna, bluefin (Cal.)	13	7.2	7	35.98	...[1]	.....[1]	..[1]	......[1]
3	Tuna, bluefin (Cal.)	110	57.6	7	42.55	9.3	8; 8	16	11; 11
3-x	Tuna, bluefin (Cal.)	41	42.4	7	41.03	7.4	9; 8	17	11; 11
3-x	Tuna, bluefin (Cal.)	81	84.9	7	45.92	16.5	8; 8	19	11; 11
7-a	Tuna, New England	39	7.9	7	39.99	6.4	10; 10	81	12; 12
7-a	Tuna, New England	46	9.2	7	41.45	8.0	9; 9	87	11; 11
9	Tuna, oriental	10	17.0	7	43.58	11.0	10; 9	65	12; 11
9	Tuna, oriental	30	51.0	7	46.55	18.7	10; 9	37	12; 11
12	Albacore	9	20.5	5	44.42	12.6	9; 9	61	10; 10
12	Albacore	9	20.5	5	44.82	13.5	9; 9	66	10; 10
12	Albacore	27	61.4	5	46.80	19.7	10; 9	32	11; 10
13	Tuna, yellowfin	40	10.7	7	45.23	14.6	9; 9	136	11; 11
14	Tuna, striped	7	9.6	5	35.62	...[1]	.....[1]	..[1]	......[1]
14	Tuna, striped	43	56.5	5	45.12	14.3	9; 9	25	10; 10
15	California bonito	20	8.4	6	34.31	...[1]	.....[1]	..[1]	......[1]
15	California bonito	140	58.0	6	45.93	16.5	10; 9	28	12; 11
16	California mackerel	630	9.8	7	44.01	11.8	10; 9	120	12; 11
17	Swordfish	180	10.1	6	45.53	15.3	9; 9	151	11; 11
18	Black sea-bass	930	7.8	7	41.98	8.5	9; 8	109	11; 11
19	Cabrilla	8600	11.0	7	40.83	7.2	9; 9	65	11; 11
20	White sea-bass	25	2.8	7	42.19	8.8	9; 8	314	11; 11
20	White sea-bass	55	6.0	7	45.74	15.9	9; 9	265	11; 11
21	Totuava	470	10.0	7	34.90	...[1]	.....[1]	..[1]	......[1]
21	Totuava	2800	59.8	7	44.47	12.7	9; 9	21	11; 11
22	Sablefish	1600	6.8	7	43.59	11.0	10; 9	162	12; 11
22	Sablefish	2300	9.8	7	45.64	15.6	9; 9	159	11; 11
23-a	'Lingcod'	3000	15.8	6	45.66	15.7	9; 9	99	11; 11
24	Bocaccio	330	8.7	6	44.46	12.7	9; 9	146	11; 11
25	Chili-pepper	4200	7.2	7	40.01	6.4	10; 10	89	12; 12
26	Wolffish	680	9.9	7	43.08	10.1	10; 9	102	12; 11
27	Basking shark	nil	5.2	7	41.94	8.4	9; 8	162	11; ·11
28	Dogfish	5800	4.0	7	42.50	9.2	9; 9	230	11; 11
29	Pollack	210	8.5	7	38.12	4.3	13; 13	51	15; 15
30	Hake	160	8.7	6	43.91	11.6	10; 9	133	12; 11
31	Sardine	270	10.7	7	43.96	11.7	10; 9	109	12; 11
Control	Cod	310	10.0	2	42.97	9.9	10; 9	99	10; 9
Control	Cod	250	8.0	2	42.08	8.6	9; 8	108	9; 8
Control	Cod	250	8.0	2	40.89	7.3	9; 9	91	9; 9
Control	Cod	250	8.0	2	42.05	8.6	9; 8	108	9; 8
Control	Cod	250	8.0	2	41.53	8.0	9; 9	100	9; 9
Control	Cod	130	4.0	2	38.81	5.1	11; 11	128	11; 11
Control	Maize oil	0	0.0	0	35.12	0.0	.....[1]	..[1]	......[1]
Sterol	Irr. ergosterol	0	200.0	..[2]	40.00	6.4	.....[2]	3.2	......[2]
Sterol	Irr. ergosterol	0	403.0	..[2]	43.30	10.4	.....[2]	2.6	......[2]
Sterol	Irr. ergosterol	0	1800.0	..[2]	46.50	18.5	.....[2]	1.0	......[2]
Sterol	Irr. cholesterol (ordinary, from spinal cord)	0	8.0	7	41.72	8.2	9; 8	103	11; 11
Sterol	Irr. 7-dehydrocholesterol	0	13.0	6	43.93	11.7	10; 9	90	12; 11

[1] Response too low for significant interpretation.

vitamin D administered per 100 gm. of ration. It records the average femur ash percentage of the group of chicks which received the oil, and the number of I.U. of vitamin D per 100 gm. of ration which theoretically would have been required to produce the observed calcification if the vitamin D had been that of cod liver oil. Finally, it shows the efficacy ratio, on the basis of cod liver oil = 100, of the vitamin D that was actually administered. Probable errors, expressed as per cent of the found values, are given for the rat assays, the chick assays,[2] and the efficacy ratios. The values for the latter were calculated from the formula,

$$\text{Efficacy ratio error} = \sqrt{\text{rat error}^2 + \text{chick error}^2}$$

In most instances, the efficacy ratio error amounted to about 11%.

At the beginning of this discussion, attention must be called to a fundamental difficulty which is encountered in attempts to give numerical expression to the efficacy ratio, or relative effectiveness, of one form of vitamin D in terms of another, rat unit for rat unit in chickens. Inspection of the master curves for cod liver oil and irradiated ergosterol, given in our paper on the assay technic with chickens, discloses that the efficacy ratio is not a constant quantity; it varies with the degree of calcification produced. Thus, one kind of vitamin D may be 80% as effective as another, rat unit for rat unit in chickens, when a certain degree of calcification is involved, and 120% when a different degree of calcification is involved. Instances of this effect are seen in table 1, where the same oil was administered at two levels, and materially different efficacy ratios were obtained. The significance of all this is, that the efficacy ratios are valid only for the degree of calcification indicated.

The first oil studied was halibut liver oil. Its efficacy ratio was 86, which means that, rat unit for rat unit, this oil was

[2] The probable error for an assay with chicks varies with the kind of vitamin D being assayed (Massengale and Bills, '36). In table 1 it is calculated as it would be if the vitamin D were always that of cod liver oil.

86% as effective as cod liver oil for producing the observed degree of femur calcification (45.42% ash) in the chicken. If we accept the conventional standard of significance, that values differing by less than three times their probable error are not significantly different, we must admit that the observed difference is not significant. Another flatfish, the round-nosed sole, gave approximately the same efficacy ratio, 81, as halibut.

Halibut liver oil is essentially a rich source of vitamin A, but it is only a few times more potent than cod liver oil in vitamin D. It occurred to us that if different kinds of vitamin D exist in nature, a good place to look for them would be in the tuna liver oils which are enormously potent in vitamin D. We examined a sample of the oil from the California bluefin tuna, Thunnus saliens. This was administered to chicks at a level of 7.2 I.U. per 100 gm. of ration, a level sufficient to produce good calcification if the vitamin D were that of cod liver oil or halibut liver oil. The resulting femur ash was so low that significant interpretation of it was impossible. Then the dosage was increased eightfold and the efficacy ratio was found to be 16.

From the fact that certain esters of calciferol are inactive, or only slightly active in healing rickets before they are saponified, it seemed possible that the anomalous behavior of the tuna liver oil was due to the existence of its vitamin D as an ester or other complex which was easily hydrolyzable by the rat but difficultly hydrolyzable by the chicken. We therefore prepared the unsaponifiable fraction of this oil, assayed it with rats, and administered it to chicks. The efficacy ratio, at two levels of administration, was 17 and 19. It follows that the low relative effectiveness of the tuna liver oil was not due, at least not in large part, to the existence of its vitamin D in unavailable form.

After the preliminary announcement of the findings on tuna liver oil (Bills, Massengale and Imboden, '34), we were informed by Dr. A. L. Emmett of Parke, Davis and Company and by Mr. Harden F. Taylor of the Atlantic Coast Fishing

Company that in their experiences the chicken efficacy ratio of tuna liver oils was not low. The recent studies of Dols ('35, '36) and Rygh ('35) also indicated that the vitamin D of tuna liver oil compared well with that of cod liver oil, rat unit for rat unit with chickens. Upon inquiry it developed that the oil studied by Emmett was from mixed species of tuna, and the oil studied by Taylor came from Massachusetts tuna. The vitamin D concentrates studied by Dols and by Rygh were from European tuna.

It happens that the name Thunnus thynnus has until recently been applied to big tunas the world over. This was the designation we accepted for the California bluefin tuna in our preliminary note, and it was based on general usage as exemplified by Jordan and Evermann (1896, '02), Jordan ('25), Ulrey and Greeley ('28), Walford ('31) and others. However, Jordan and Evermann ('26) and Jordan, Evermann and Clark ('30) have in recent years changed their original view that the genus Thunnus is represented by only one species, T. thynnus. They now provisionally recognize eight species, of which we are concerned with the California T. saliens, the New England T. secundodorsalis, the Japanese T. orientalis, and the European T. thynnus. Knowledge of the tunas is so imperfect that this reclassification ventured by Jordan and Evermann ('26) on the basis of slight differences in photographs and drawings has not met with general acceptance by ichthyologists. However, our findings strongly support the reclassification, and incidentally introduce a new, biochemical basis for taxonomy.

The New England tuna, Thunnus secundodorsalis, lost its identity under T. thynnus in Jordan and Evermann (1896, '02), Jordan ('25) and Bigelow and Welsh ('25). It was later distinguished by Jordan and Evermann ('26) and Jordan, Evermann and Clark ('30) as above indicated. The livers of this species yielded an oil which contained approximately the same amount of vitamin A as the oil of the California bluefin. However, the vitamin D content was much lower than that of any California bluefin tuna liver oil that

we have encountered in the examination of many large and small samples. The outstanding difference, as shown in table 1, was that the efficacy ratio of the vitamin D was high, 84 —essentially in agreement with the private experiments of Mr. Taylor.

We have not had the opportunity to examine the liver oil of the European tuna, Thunnus thynnus, but if the findings of Dols ('35, '36) and Rygh ('35) can be accepted, this species possesses vitamin D similar in kind to that of T. secundo-dorsalis.

The Japanese bluefin tuna, 'maguro,' Thunnus orientalis, was T. thynnus in Jordan and Evermann (1896) and Jordan, Tanaka and Snyder ('13). It was recognized as a separate species by Kishinouye ('23) and Jordan and Evermann ('26). The characteristic feature of its vitamin content is that the effectiveness of its vitamin D, rat unit for rat unit on chickens, is between that of T. saliens and T. secundodorsalis. At two levels of administration, efficacy ratios of 65 and 37 were obtained. These are in agreement with the findings of Black and Sassaman ('36).

The albacore, or long-finned tuna, is Thunnus germo in the critical study by Kishinouye ('23); Germo germo in most of the check lists. The data in table 1 indicate that the chicken effectiveness of albacore liver oil, per rat unit, is similar to that of oriental tuna. Early in our work, we once observed an unusually low efficacy ratio, 12, in albacore liver oil. In that instance, however, there was some doubt as to the composition of the test sample. The findings were therefore not included in the table.

Leaving the genus Thunnus, we now consider the data for related tunas and other scombroid fishes. The vitamin D of the liver oil of yellowfin tuna, Neothunnus macropterus, had an efficacy ratio of 136, which appears to be significantly greater than the ratio 100 for cod liver oil. Black and Sassaman ('36) found a value of 63 for yellowfin tuna, but it is to be noted that their livers were from Japanese fish, whereas ours were from fish caught off the coast of Mexico

and Central America. The difference may be one of environment, or possibly of species.

The liver oils of Mexican striped tuna, Katsuwonus pelamis, and the closely similar California bonito, Sarda lineolata, failed to produce mentionable calcification in chickens when a rat unitage was administered that should have done well had the vitamin D been that of cod liver oil. At much higher levels, efficacy ratios of 25 and 28 were obtained. Black and Sassaman ('36) reported a ratio of 46 for Japanese striped tuna. The California mackerel, Pneumatophorus diego, is a small relative of the tunas. Its liver oil showed an efficacy ratio of 120, which is not significantly above the ratio 100 for cod liver oil. Black and Sassaman's similar findings for Japanese mackerel cannot be compared with ours, because Japanese mackerel is definitely a different species.

The liver oils of five miscellaneous percomorphs, all important new vitamin sources, were next investigated. Swordfish gave an efficacy ratio of 151, distinctly higher than that reported by Black and Sassaman ('36). Black sea-bass, Stereolepis gigas, which shares with its Asiatic cognate, Stereolepis ishinagi, the distinction of being the richest known source of vitamin A, gave an efficacy ratio of 109. Cabrilla, Epinephelus analogus, which is classed in the same family (Epinephelidae) with the black sea-bass, gave an efficacy ratio of 65. White 'sea-bass,' Cynoscion nobilis (which properly is not a sea-bass), gave an efficacy ratio of around 300, the highest observed in any of the twenty-five species which were compared with cod. Totuava, which is classed in the same family (Otolithidae) with the white sea-bass, gave an efficacy ratio of only 21.

The liver oils of four fish in the order Cataphracti were investigated. Sablefish liver oil at two levels of administration to chicks gave efficacy ratios of 162 and 159. Lingcod gave 99. Bocaccio and chili-pepper gave 146 and 89, respectively.

Our study was completed with six other oils from species in five zoölogical orders. The liver oil of wolffish rated 102.

The basking shark, which is an extraordinarily poor source of vitamins, gave an efficacy ratio of 162. Dogfish rated 230. Two codfishes, whose liver oils contribute materially to the 'cod liver oil' of certain localities, were investigated. The pollack, Pollachius virens, rated 51. The hake, Urophycis chuss and U. tenuis (species so similar that few fishermen can distinguish them), rated 133. Liver oils of the pollack and these particular hakes are legally cod liver oil; the difference in efficacy ratios would suggest that the potency of cod liver oil, as ordinarily expressed after assay with rats, is not an infallible measure of the effectiveness of cod liver oil for chickens. The oil of the sardine, Sardinia caerulea, was the only oil studied which was not wholly a liver oil. The specimen was a typical commercial sardine oil, enormous quantities of which are used in the poultry industry. We found the efficacy ratio to be 109, a value not significantly different from the value 100 for cod liver oil and quite in keeping with the experience of practical poultrymen.

In coming to the conclusion that the data summarized in table 1 demonstrate the existence of two or more kinds of vitamin D in fish oils, we point out the following. The number of species of fish investigated was large. In an ample number of instances the differences observed were much greater than the probable errors of assay. The extreme difference was between the oils of California bluefin tuna and the so-called white sea-bass, the latter being about eighteen times as effective for chickens, per rat unit, as the former. Even with allowance for the difficulty already mentioned in regard to the numerical evaluation of efficacy ratios, and the necessarily indirect comparisons via cod liver oil, the difference observed between the liver oils of white 'sea-bass' and California bluefin tuna is far greater than the probable errors involved.

Inspection of the efficacy ratio column in table 1 reveals no clustering of values around 100. Were it otherwise, i.e., if there were numerous values near 100, and gradually fewer above and below 100, one would suspect that an overlooked source of error was at work. As it is, the scatter of values

seems wholly erratic, and indicative of the likelihood that in a larger series of fish oils, even greater differences in relative effectiveness would be observed.

The possibility of suppressed availability of the vitamin D in certain oils would seem to have been discounted by the experiment with the unsaponifiable fraction of the first tuna oil. The possibility that free fatty acids in the oils played any role is eliminated by the fact that most of the oils, including those of highest and lowest relative efficacy, were practically neutral. Moreover, most of the oils were greatly diluted before they were mixed with the diet, so that the vehicle of the vitamin was principally maize oil. Lastly, it is obvious from table 1 that there is no relation between the vitamin A content of the oils and the efficacy ratio of their vitamin D.

Our findings are explicable on the basis that two (or more than two) kinds of vitamin D exist in fish oils, the proportions varying in the different oils. It now seems unlikely that any particular fish oil, such as cod liver oil, contains one kind exclusively. No fish oil has been found with as low an efficacy ratio as irradiated ergosterol (calciferol). The latter has an efficacy ratio of from 1.0 to 3.2 when administered at dosages sufficient to produce degrees of femur calcification comparable with those given by the fish oils under the conditions of our experiments. It is conceivable, therefore, though not especially probable, that calciferol is the component of the fish oil vitamin D complex which has the lower efficacy ratio. At the other extreme, no artificial kind of vitamin D has been found with as high an efficacy ratio as certain fish oils, particularly as white sea-bass liver oil.

In collaboration with Dr. F. G. McDonald we have undertaken to determine the efficacy ratios of all the known artificial forms of vitamin D. It is especially desired to ascertain whether any of these has as high an efficacy ratio as white sea-bass liver oil. If none has, then the component of the fish oil vitamin D complex which has the higher efficacy ratio will have to be regarded as a new form of vitamin D—the ninth,

in fact. So far, we have discovered that irradiated 7-dehydro-cholesterol is the artificial form of vitamin D with the highest efficacy ratio. For the production of certain degrees of calcification in the chick it is as effective, rat unit for rat unit, as cod liver oil. Irradiated ordinary cholesterol is similar in efficacy ratio to irradiated 7-dehydrocholesterol. Irradiated sterols of the higher plants, and also, according to McDonald's findings, irradiated 22-dihydroergosterol, are distinctly lower in efficacy ratio than these, but higher than irradiated ergosterol. Details of our studies with the above-mentioned irradiated sterols, and of still other forms of vitamin D, will be given later.

SUMMARY

The liver oils of twenty-five species of fish were assayed in comparison with cod liver oil on rats and chickens. Rat unit for rat unit, some resembled cod liver oil, several were definitely less effective, and a few were more effective than cod liver oil. The oils which were relatively the least effective were those from bluefin tuna of California, oriental tuna, striped tuna, bonito, albacore and totuava. The relatively most effective oil was that from the white sea-bass of California. The maximum observed difference in relative effectiveness was about eighteen times, a difference much greater than the probable errors of assay.

As possible causes of the differences, the following were eliminated: Existence of vitamin D in conjugated forms of unequal availability, synergism or antagonism of vitamin A, presence of free fatty acids, and the nature of the oily vehicle. It was concluded that the differences were due to the existence of two or more forms of vitamin D in the oils.

The possible relation of the fish oil vitamins D to certain artificial forms, particularly irradiated ergosterol and irradiated 7-dehydrocholesterol, was discussed. The relative effectiveness of irradiated ergosterol was lower than that of any fish oil. The relative effectiveness of irradiated 7-dehydrocholesterol was about the same as that of cod liver oil or irradiated ordinary cholesterol, but inferior to that of white sea-bass liver oil.

Oils of related species, particularly the several tunas, differed widely in relative effectiveness. The findings support the view that the big tunas formerly classed as Thunnus thynnus actually comprise several species, of which we have studied T. saliens, T. orientalis and T. secundodorsalis.

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SOME QUANTITATIVE STUDIES ON REFECTION IN THE RAT [1]

EUNICE KELLY AND HELEN T. PARSONS

Department of Home Economics, Universtiy of Wisconsin, Madison

TWO FIGURES

The general theories concerning the nature of refection have been summarized by Browning ('31) and by Bliss ('36). Opinion in America concerning the phenomenon has been divided. Mendel and Vickery ('29) failed to obtain it on standard rations and raised the question of a possible role of residual vitamin B in the diet itself. On the other hand, its occurrence has been reported by Kon ('31) and Lepkovsky[2] from Professor Evans' laboratory; by Parsons, Kelly and Hussemann ('33), and Parsons and Kelly ('35); and by Bliss ('36). Each of these interpreted this occasional growth of rats on vitamin B-low rations in agreement with the general

hypothesis first formulated by Fridericia ('26). In contrast with these, on the other hand, the assumption was made by Whipple and Church ('35) that the phenomenon is essentially dependent on the small amount of non-extractable fat in raw starch which is made available to the animal when the starch granule is ruptured in the digestive tract; this theory grew out of their observation that the 'content' (i.e., concentration) of the anti-beriberi factor in the feces of rats on vitamin B-low rations in their experiments was directly correlated with the amount of lard in the diet.

<div align="center">EXPERIMENTAL</div>

First occurrence of refection in this laboratory

Refection was first observed in 1931 in this laboratory during the progress of a rough assay of some foodstuffs for their vitamin G content. Potato starch was tested both as a crude preparation and as a commercial sample by substituting it for 35% of cornstarch when growth had ceased after 2 weeks on the following ration; purified casein 18, Osborne and Mendel's[3] salt mixture 4, rice polishings 6, cod liver oil 2, sucrose 35 and cooked cornstarch 35%. There was a slow gradual gain after which growth again ceased. Most of the group was discarded at this time but not before a part of them had made a sudden unexpected gain. Of these, three were retained because a few days before the upward turn occurred, large white feces had been noticed on the papers under the cages. Average gains of 2.5 gm. per day were made by these rats for several weeks after which the rice polishings were removed from the ration of one rat and reduced to 2% in the ration of another; the former rat lost weight steadily and was discarded, but the latter continued to gain until it weighed 332 gm. At this point cooked potato starch was substituted for the raw, whereupon the rat lost weight rapidly and died. The condition was thereafter produced in a succession of animals on the diet on which this rat had thrived: casein 18, salt mixture 4, rice polishings 2, cod liver oil 2, sucrose 24 and raw potato starch 50. This will be referred to as ration 1.

[3] Osborne and Mendel ('19).

Characteristics of the condition secured on rations containing rat potato starch

The feces of the presumably refected rats were bulky with occluded air spaces and were loaded with undigested starch grains clearly visible under the microscope.[4] The color ranged from creamy white to brownish tan; the degree of whiteness was not well correlated either with the concentration of vitamin B in the refected feces or with the rapidity of the rat's growth; in fact, after the condition was established, large brownish-tan feces seemed to be as good an indication of its persistence as were strikingly white feces. It is conceivable that the latter are rather an indication of the condition which presumably initiates refection, i.e., the passage of a large amount of undigested starch granules into the coecum, thus providing a substrate for the multiplication of organisms, but that when these are well established their activity may often result in the splitting of the greater part of the raw starch entering the coecum, thus yielding brown rather than white feces albeit with an accompanying production of a large amount of vitamin B.

Attempts to establish refection on purified rations containing raw potato starch but no other possible source of the vitamin B-complex were not successful, contrary to the experience of others; although white feces appeared as early on these purified rations as on those containing rice polishings, the animals did not thrive; only when small amounts of some substance such as rice polishings or autoclaved yeast were included in the ration was growth obtained. Preformed vitamin B seemed not to be responsible for the effects of these additions inasmuch as the yeast had received thorough heat treatment. Young rats at weaning, fed a standard vitamin B-low ration containing 6% of autoclaved yeast as a source of the vitamin B complex, until the body weights declined, began to grow rapidly and to show other signs of refection at varying intervals after 50% of potato starch was substituted for an equal amount of sucrose in the ration and the

[4] Samples of these refected feces were exhibited in a demonstration given at the meeting of the American Society of Biological Chemists at Cincinnati, 1933.

feces eliminated were made available to the rats. Reproduction was successful in the first generation of these rats but not in the second. Assays showed that the feces contained considerable, although variable, amounts of vitamin B.

The reason for the lack of digestion of raw potato starch eliminated in feces containing diastase, a phenomenon recorded by various observers, is not known. In a few of the present experiments the progress of the digestion of the potato starch with saliva and malt diastase was followed by observations under the microscope. After 72 to 144 hours many of the larger grains were fissured or broken but the smaller grains were apparently untouched, in harmony with the observations of Whymper ('09) on the relative resistance of the latter to the action of various agents. No qualitative difference was noted between the behavior of the starch of the ration and that of the grains remaining undigested in the feces. This is in harmony with the results of Bliss ('36). The experiment suggested, however, that the peculiar resistance to digestion which the potato starch was observed to acquire by passage through the digestive tracts of the refected rats in Fridericia's laboratory (Fridericia and associates, '27–'28, and Nathan, '34) may have been due to a disappearance of most of the large starch grains in the digestive tracts of the rats either by the action of the digestive juices or of organisms, thus leaving the starch of the feces made up much more largely of small resistant grains than was the original starch of the ration.

Meteorism was intense in the rats on raw potato starch rations, even causing the death of animals that had been on the ration for some time and were gaining rapidly.

Characteristics of the condition secured on rations containing gelatinized potato starch

It was desirable to test the performance of rats on ration 1 when the potato starch of the ration had been subjected to sufficient moist heat to render the starch grains readily digestible thus presumably reducing the amount of food material reaching the coecum, but at the same time avoiding any

detectable destruction of vitamin B. Bare gelatinization of the starch seemed to answer these requirements; this was accomplished by quickly combining a cold water suspension of the starch with sufficient hot water to result either in a temperature of 63 to 65° which was held for 5 minutes, or a temperature of 70 to 73° which was followed by as rapid cooling of the paste as possible in thin layers. These methods are comparable with that of Bliss ('36). As in the experiments with raw starch, the technic was also followed here of feeding 3% of refected feces for 9 days. Of the group of forty rats started on this ration, none gave those evidences of refection that had been observed in the group on raw starch in regard to growth or size and color of feces. The individual gain in weight during the first 3 weeks averaged 17 gm. for the group. The feces were uniformly small, hard and very dark. Many of the group were discarded when growth ceased at some time before the end of the third to fifth week. Of the remainder, fifteen rats were observed to develop quite definite manifestations of a nutritive disorder, from the twenty-fourth to the forty-third day of the experiment. These manifestations included a huddled posture, sharply bent back, persistence in clutching and clinging to the wires of the cage, insecure gait, and difficulty of balance with a tendency to turn in circles, to hold the head on one side and to fall. Two rats in an extremely serious condition made a swift and apparently complete recovery after injections of Eli Lilly and Company's[5] concentrate of vitamin B_1. Inasmuch as the symptoms were so closely similar to those described for vitamin B_4 deficiency, a preparation of this vitamin, kindly furnished by Dr. C. A. Elvehjem, was fed by mouth to two rats but gave slight or even doubtful benefit. It seems quite possible that the deficiency was multiple but certainly the lack of vitamin B was pronounced.

Evidence will be cited later in more nearly quantitative experiments, that there was no significant destruction of vita-

min B during gelatinization if, indeed, this vitamin occurred in appreciable concentration in the potato starch used.

Comparison of the vitamin B in the feces of rats on equalized intakes of rations containing either high or low fat and either raw or gelatinized potato starch

Although the performance of rats on vitamin B-low rations containing raw potato starch was clearly superior to that on gelatinized, it seemed to us that one aspect of the problem had not been considered by the objectors to Mendel and Vickery's theory when they argued that all animals would behave alike if the growth in refection were attributable to residual vitamin B in the ration; this aspect was that rats on potato starch rations show wide variations in regard to the amount of potato starch eliminated in their feces, which, as Fridericia has shown may amount to as much as 62% of the weight of the feces on raw starch rations. This fraction of potato starch, not being absorbed and utilized by the body, would presumably involve a corresponding increase in the food intake of these rats in comparison with rats not eliminating starch in their feces and might thus conceivably account for an unequal intake of vitamin B if residues of this vitamin were in fact present in the ration. To test this point, the following experiment was undertaken whereby the effects of making the starch readily digestible and of adding a fat to the ration were separated from the effects of inequalities of food intake by carefully equalizing the latter factor.

Details of the experiment. Two groups of rats weighing about 45 gm. were fed ration 1 containing raw potato starch and gelatinized potato starch, respectively; the rats on the contrasting diets were paired, the food intake of the pair being controlled by the pacemaker and varied from day to day. With the results of this experiment as a guide in regard to the intake of food which it was possible to achieve, other groups of six rats each were started with rigid daily food intakes of 3.5, 4, 4.5 or 6 gm. For these, both the raw and the gelatinized starch rations were varied by the substitution

of 10% of lard for an equal weight of sucrose in the rations
of certain groups. Many rats had to be dropped from each
group because of failure in food intake, and larger rats were
substituted, care being taken to keep the average weights
of the various groups approximately equal. In the case of
the groups on gelatinized starch, only those on 4 gm. or less
daily intake of the ration containing lard included as many
as six rats at the end of the period.

The vitamin B-content of the feces eliminated by these donor
groups over equal periods of time were tested by feeding
them to an equal number of assay rats either as intact indi-
vidual daily collections or after mixing, pulverizing, and re-
dividing the total 30 days' feces collection of a given donor
rat. During a preliminary 9-day period, 3% of refected feces
were included in the ration of the donor rats in order to
equalize the opportunity for the transmission of the agent
of refection to the rats in the various groups in case this
precaution should be needed; the collections of feces of the
donors were discarded during these 9 days. No difficulty
was experienced in securing consumption of the feces doses
by the assay groups. Control groups of assay rats were
fed the basal ration alone and with the addition of 2 inter-
national units of vitamin B per day supplied in weighed
portions of a yeast standardized by Dr. E. M. Nelson of the
United States Department of Agriculture, Washington, D. C.,
against the international vitamin B preparation of the Health
Committee of the League of Nations.

It was considered essential to assay some representative
body tissues of the donor rats for their content of vitamin B
to furnish evidence as to whether or not a possibly high content
of vitamin B in the feces might represent losses of body stores
of this vitamin, or a low content, uncontrolled coprophagy with
a storage of the vitamin in the tissues.

The body organs which were assayed were taken from the
larger rats in each group; they comprised the liver, kidneys,
spleen, heart and lungs. These were removed from the body
after the donor rat had been bled from the jugular vein

under anaesthesia and killed. These tissues were then minced with scissors, dried as one preparation from each rat at room temperature before an electric fan and pulverized with a mortar and pestle. In preliminary experiments, doses of 0.7 gm. of such dried preparations were given to assay rats which had been losing weight for 3 to 4 days on the vitamin B-low ration, and the dosage was repeated when the body weights had again decreased to the same point after the gain due to the dosage. In this way, a very good estimate could be made of the most favorable concentration at which to feed the later samples, as a part of the vitamin B-low ration, to bring out comparisons between groups. Thereafter, a composite sample from three donor rats was fed incorporated in the ration of one assay rat in a 15-day test period. This time interval was considered fully adequate in view of the observations of Knott and Schultz ('36) on a 10-day vitamin B assay.

Although none of the rats was observed to practice coprophagy at any time, the harness of Page ('32) was tested but was found to be unsuitable for our purpose. It obviously could not be used for the rats with intense meteorism; even for the others it was discarded since it was necessary to draw it so tightly as to seem to cause severe discomfort and interfere with food intake.

Results. It may be seen in figure 1 that the growth of the donors on any one level of food intake was quite comparable for the most part, but it is notable that growth was somewhat poorer on the raw then on the gelatinized starch ration with an equal calorie intake, possibly due to loss of some of the starch in the feces from the former, resulting in a lower actual intake of energy.

When one examines the growth of the assay rats in figure 2 and table 1 there is seen to be a striking difference between the average vitamin B-content of the feces produced on a ration containing 50% of gelatinized potato starch with 10% of lard (group 1) and that on an equal intake of a comparably fat-rich ration containing raw potato starch (group 2). Some curves of group 2 were equal to those of rats fed 2 international units

of vitamin B daily, whereas no rat in group 1 made any sustained gain. That this difference between groups 1 and 2 is not attributable to an unequal content of performed vitamin B in the ration itself dependent on a destruction of the vitamin during the gelatinization of the potato starch is clearly proven by the fact that the feces of one of the donors on raw potato starch (group 2) were no richer in vitamin B
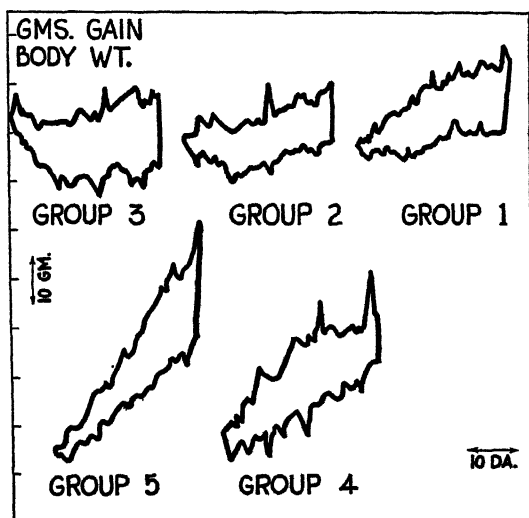
Fig. 1 Solid areas represent combined growth curves of sets of donor rats on the following diets: Group 1, 4 gm. daily intake of ration 1 with gelatinized potato starch + 10% lard. Group 2, 4 gm. daily intake of ration 1 with raw potato starch + 10% lard. Group 3, 4 gm. daily intake of ration 1 with raw potato starch. Group 4, 6 gm. daily intake of ration 1 with raw potato starch + 10% lard. Group 5, 6 gm. daily intake of ration 1 with raw potato starch.

than those of some of the donors on gelatinized. To determine whether or not the failure of this one assay rat was due to an inability to grow, it was fed 0.5 gm. per day of feces from another donor rat; the prompt and vigorous response in growth, represented by the dotted line, gave ample evidence that the previous lack of growth was in fact due to the low content of vitamin B in the first donor's feces which had been fed to the assay rat in a 0.54 gm. daily dosage. Presumably
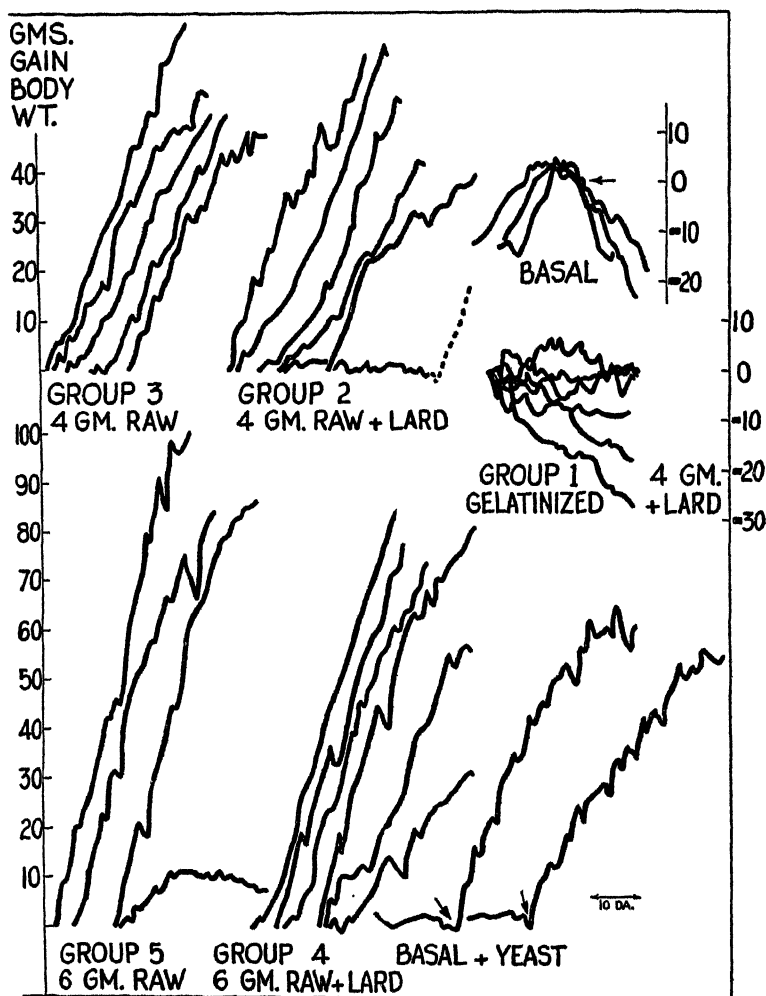
Fig. 2 Assays of the vitamin B content of daily collections of feces of donor rats shown in figure 1. The preliminary depletion period used for all assay rats is represented in this figure only for the groups in the negative and positive control experiments, respectively; for the others the curves begin with the feeding of the test doses. Note the strikingly greater elimination of vitamin B in the feces of the rats on raw potato starch in comparison with those on gelatinized, and the lack of effect of introducing 10% of lard into the ration. ←, marks the standard point of depletion of vitamin B in the negative and positive control experiments which was chosen for the beginning of dosage in the assays. ----- in the last curve in group 2 indicates a change in dosage, the feces of another donor rat being substituted for those which failed to promote growth.

this particular donor rat had not become 'refected' on raw starch as was evidently true also for one donor in group 5.

Some assay rats were fed feces in uniform daily amounts that had been produced on a 3.5 gm. level of the starch rations.

TABLE 1

Showing details of the assays for vitamin B in the feces and organs of rats on equalized intakes of potato starch rations

IDENTITY OF GROUPS AND RATIONS	INITIAL BODY WEIGHT OF DONOR RATS		TOTAL WEIGHTS DRY ORGANS FED IN 15-DAY PERIODS, THREE DONOR RATS TO EACH SET ORGANS	ASSAY RATS		AVERAGE WEIGHT DAILY COLLECTIONS FECES FED
	Average for total group contributing feces	Average for total group contributing organs		15-day gain in body weight in assay rats fed each lot organs	Average 30-day gain body weight assay rats fed feces	
1 Gelatinized starch + lard 4 gm. intake	gm. 66	gm. 74	gm. 3.5 4.7 3.4	gm. — 16 — 12 — 25	gm. — 10	gm. 0.16
2 Raw starch + lard 4 gm. intake	60	81	4.2 4.1	6 — 9	44	0.54
3 Raw starch low fat 4 gm. intake	66	84	3.7 4.0	12 17	56	0.49
4 Raw starch + lard 6 gm. intake	65	85	6.7 5.9	29 0	73	0.74
5 Raw starch low fat 6 gm. intake	63	86	5.7 5.6	29 3	70	0.89

The assay rats fed 0.5 gm. of feces from rats eating gelatinized potato starch showed practically the same rate of growth as the assay rats fed 0.5 gm. of feces produced on equal amounts of raw starch; these portions of feces collected on gelatinized starch represented as much as triple the length of time of the

feces collections on raw starch. Hence the elimination of vitamin B was three times greater on raw starch than on gelatinized.

The magnitude which is evident in the variations in the vitamin B content of the feces within any one of the groups of rats fed uniform amounts of a raw potato starch ration (fig. 2) tends to substantiate Fridericia's hypothesis of the nature of refection rather than that of Mendel and Vickery; an unequal consumption of 'residual' vitamin B is ruled out as a cause for the sharp difference between the averages in groups 1 and 2 and, on the other hand, the range in the fecal elimination of vitamin B in group 2 would be expected if it depends upon a synthesis in the intestine.

Inasmuch as a consumption of feces was not rendered entirely impossible in these experiments, it might be postulated that this had been practiced by group 1 of the donors to a greater extent than by group 2 and thus accounted for the difference in the apparent vitamin B-elimination between the two; this might seem the more probable because the average weight of the daily collections of feces for group 2 was about three and a third times that of group 1 (see table 1). However, if coprophagy had accounted for a disappearance of vitamin B from the feces collection of group 1, the vitamin B-content of the organs of this group would presumably have been correspondingly higher than in group 2. Table 1 shows this not to have been the case as the organs of group 2 were richer in vitamin B than those of group 1; therefore, coprophagy is ruled out as a factor of significance in the low content of vitamin B in the feces of the group fed gelatinized potato starch. It is not necessarily eliminated, however, as a source of some of the store of vitamin B in the organs of the rats on raw starch; the experiment therefore does not contribute evidence as to whether the refected rat obtains vitamin B through direct absorption of the vitamin at the site of synthesis, presumably in the coecum, or through a consumption of some of the vitamin rich fecal material.

The results in figure 2 and table 1 on the assay of the feces of groups 2 and 4 in contrast to groups 3 and 5 of the donor rats show unmistakably that the presence of lard in the ration had no measurable influence in increasing the total content of vitamin B in the rats' feces under the conditions of these experiments, hence the assumption of Whipple and Church that their observation on the concentration of vitamin B in the feces of their animals on rations varying in fat content explains the phenomenon of refection is without justification. Caution is necessary, however, in making interpretations from the present results in regard to other relationships of the fat of the diet to the metabolism and elimination of vitamin B inasmuch as certain variables were introduced by the nature of the study. It is worthy of comment, nevertheless, that in these few assays no tendency is apparent for the body organs studied to show a higher content of vitamin B when the restricted fat of the diet is increased by 10% of lard; the effect of synthesis in the intestine may well have masked such a tendency if it was present. However, the question might be raised as to whether or not the magnitude of the postulated effect of dietary lard in checking the losses of vitamin B from the body stores of rats in the experiments of Evans and Lepkovsky ('35) may not have been influenced, in some degree, by the effect on the intestinal flora of the much larger food intake of one of the fat-fed groups in contrast to the fat-low. This question arises particularly since these authors found that fecal elimination of the vitamin did not at all parallel the rate of its disappearance from the tissues. In the present experiments the effect of the amount of food intake is seen in that the rats on the 6-gm. intake tended to show a greater average fecal output of vitamin B than those on 4 gm. In view of the very wide variations within the individual groups, this difference, although occurring on rations which were admittedly not purified, seems to fit more logically with the assumption that it is attributable to a tendency for more food to reach the coecum than it does with the idea that the trace of preformed vitamin B introduced

into the ration by the 2% of rice polishings and possibly by the potato starch is significant. In the experiments of Evans and Lepkovsky one would be inclined to assume that the exclusion of starch from the ration ruled out all possibility of an influence of food intake on the degree of synthesis of the vitamin in the intestine and yet this is not entirely certain. The rations containing autoclaved acidified cornstarch in the experiments of Guerrant, Dutcher and Tomey ('35) appear to have supplied conditions for the synthesis of vitamin B in the digestive tract somewhat comparable to those in raw potato starch rations, although no explanation for this is at hand.

In some determinations of the vitamin B concentration in the feces of stock rats, very decided fluctuations were found from time to time although the composition of the ration remained uniform as far as was known; presumably these fluctuations were due to a varying synthesis of the vitamin rather than to a varying excretion. The uneven response obtained on a given stock ration at different times or in different laboratories may occasionally be due to a fluctuation in the synthesis of some factor rather than to variations in the foodstuffs or a gradual depletion of a factor in succeeding generations.

Results with starches other than potato. Paired feeding experiments (Seibert, '36) similar to those just described for potato starch but with corn and rice starches substituted in its place showed that no more vitamin B was eliminated in the feces of rats on these latter starches when the starch was fed raw than when it was gelatinized. This is in harmony with the fact that negative control experiments performed recently in this laboratory in vitamin B assays in which raw cornstarch was employed, have shown no variation from the results of former years, even though carried out in close proximity to refection experiments. The present studies give greater evidence for the etiological importance of the amount of unabsorbed food reaching the coecum of the rat than for the infectious nature of refection, postulated by others.

SUMMARY

1. The occurrence in rats on raw potato starch rations of a phenomenon with the characteristics of the 'refection' observed by Fridericia and others is described.

2. Gelatinization of the potato starch at temperatures not significantly destructive of vitamin B prevented the occurrence of refection.

3. On uniform intakes of a ration high in potato starch, rats tended to eliminate about three times as much total vitamin B in the feces when the starch was raw as when it was gelatinized.

4. The non-extractable fat of the starch is not a significant factor in the phenomenon of refection.

5. Some applications of these results to various problems in nutrition are suggested.

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A COMPARISON OF HEATED CASEIN WITH EX-TRACTED CASEIN IN THE BASAL DIET FOR THE DETERMINATION OF VITAMIN A [1]

E. NEIGE TODHUNTER

College of Home Economics, State College of Washington, Pullman

The influence of heat on milk proteins has been widely studied. Greaves and Morgan ('34) showed that the lowered nutritive value of casein which had been heated was due to definite changes in the lysine and histidine fractions. Chick and associates ('35) reported that heating casein at 150°C. for 66 hours reduced the biological value from 64 to 45 when fed as 5% of the diet, and reduced the digestibility from 93 to 73%. Schultz, Seegers and Mattill ('35) observed that casein treated by heat or extraction does not permit optimum growth in experimental animals and they found that prolonged heating seemed to destroy or alter the nutritive value more completely than heating for a short period at a higher temperature. Fairbanks and Mitchell ('36) found the proteins of milk very sensitive to heat with respect to their value in nutrition; in the early stages of heating skim milk powder, cystine was destroyed but in the later stages when scorching occurred, the destruction of lysine exceeded that of cystine.

Casein extracted with boiling alcohol has been widely used as a source of protein in the vitamin A-free basal diet for rats. Giddings and Swim ('34) have pointed out the variability in the reaction of rats in vitamin A experiments and the lack of a standard method of extraction of the casein.

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In a study of a number of methods of extracting casein they showed that the Sherman and Smith ('31) method of extraction resulted in the removal of the maximum amount of vitamin A from the constituents of the basal diet.

Fraps ('31) in his vitamin A studies used casein which had been heated to 110°C. for 24 hours and reported that results were satisfactory when this casein was compared with that purified by extraction, but he presented no experimental evidence on this point.

Casein heated for 7 days at 110°C. has been recommended by Potter ('32) as a source of protein, free from vitamin A. Potter reported no differences in experimental findings when using heat-treated casein in place of alcohol-extracted casein. Her studies were not, however, made on litter mate controls and Coward, Key and Morgan ('33) have shown that variable responses may be obtained in animals of the same laboratory from year to year and even from litter to litter. It was therefore considered advisable to verify Potter's ('32) work, eliminating possible seasonal or hereditary differences and making direct comparison of the response of a group of animals receiving casein, heat-treated according to the method of Potter ('32), with the response of another group receiving casein, alcohol-extracted according to the method recommended by Giddings and Swim ('34). Conclusive evidence of the reliability of the use of heat-treated casein in vitamin A-free diets would be of value to those laboratories that lack the equipment for alcohol extraction of casein, and would also provide a cheaper method of preparation.

PROCEDURE

Albino rats of known nutritional history but from two different stock colonies were used in this study. Fifty-six rats were placed upon two experimental diets which were alike except for the treatment of the casein.

· The vitamin A-free basal diet consisted of:

	%
Casein (freed from vitamin A)	18
Dried yeast [2]	10
Salt mixture (Osborne and Mendel)	4
Cornstarch	68
Each rat received 3 drops of viosterol per os weekly	

Diet I consisted of the above, using casein which had been heated for 7 days at 110°C. in a thermostatically controlled electric oven. The casein was heated in shallow pans, being stirred twice daily and systematically rotated to insure uniform heating throughout.

Diet II was similar to diet I except that the casein had been extracted with alcohol as follows: 1200 gm. of casein were placed in a 6-liter balloon flask with 3000 cc. of 95% alcohol and refluxed on a boiling water bath for 1 hour. An electrically driven stirring device was used to keep the casein agitated throughout the entire time. At the end of the hour the casein was filtered with suction, returned to the flask and 2800 cc. of boiling alcohol added while stirring. This was refluxed for 1 hour, filtered with suction, and again refluxed with 2800 cc. alcohol for 1 hour. The casein was filtered, washed on the filter with 3000 cc. boiling alcohol which was removed by suction and then spread out to dry under an electric fan at room temperature. Reclaimed alcohol was used for the first two extractions and new alcohol for the last extraction and washing.[3]

The rats were placed on the vitamin A-free diets at 21 to 28 days of age and weighing 35 to 50 gm. They were kept in individual cages throughout the experiment. Weight records of the animals were kept weekly for the first 3 weeks, after which time weighings were made every other day until the death of the animal. Distilled water was supplied at all times, the basal diet was always accessible, and records of food consumption were made each week. The time taken to

[2] From the Northwestern Yeast Company, Chicago, Illinois.
[3] We are indebted to Mr. Harvey Murer, research dairy chemist of the Washington Agricultural Experiment Station for preparing the extracted casein.

deplete the animals of their body stores of vitamin A was recorded in all cases and was determined by stationary or declining weight for 1 week and/or xerophthalmia. All animals were examined for external signs of vitamin A deficiency until death, and at autopsy the nose, middle ear, glands at the base of the tongue and neck, intestinal tract, kidneys, lungs and bladder were observed for macroscopic signs of infection.

The animals used were divided into three groups as follows:

Group A was composed of rats having a different hereditary and dietary background from the succeeding groups. Males only were available for use in this group, the females being used for fertility studies. The rats in this group were divided into two sub-groups and fed diets I or II; the average initial weight of rats on each diet was the same.

Group B was our regular stock colony animals, and both males and females were used. The rats in this group were evenly divided into two sub-groups of comparable weights and sexes and were fed diets I and II.

Group C was composed of a small number of rats of the same stock as group B. These animals were equally distributed between diets I and II and after depletion of their body stores of vitamin A they received in addition to the basal diet, 3 drops daily of cod liver oil. This quantity was considered sufficient to promote normal growth in the rat if all other dietary constituents were adequate. This supplementary feeding was continued for a period of 60 days at which time all animals were chloroformed and autopsied.

RESULTS AND DISCUSSION

In group A the depletion period averaged 28.6 days for the animals on diet I and 29.3 days on diet II with average weights of 98.5 gm. and 97.0 gm., respectively, at this stage. The total survival period, from the time the animals were placed on the vitamin A-free diets until death, together with the weight change during this period are here considered to be the best indications of the comparative value of the two

diets and therefore of the two types of casein. With diet I the average survival was 40 days with a final weight of 79.7 gm. and on diet II, 44 days and 73.7 gm.

For group B, the data for the males and females are recorded separately. The males were of greater initial weight than those in group A, they showed a longer depletion period of 44.9 days on diet I, and 44.3 days on diet II, and their average weights at depletion were greater, being 181.8 gm. and 179.3 gm., respectively. The total survival on the two casein diets was strictly comparable being 65.9 days and 65.0 days, with final weights at death of 131.7 gm. and 116.6 gm.

While the data secured with the two diets are in close agreement, the results from the two groups are different thus emphasizing the influence of hereditary and dietary background on the growth and survival of vitamin A-deficient rats. Such a difference also emphasizes the necessity of making simultaneous tests with a standard of reference and the unknown test food at all times in quantitative vitamin A determinations.

The females of group B showed similar weight responses, depletion time, and survival on the two diets, their weights, however, were lower, and the survival period longer than for the corresponding males.

The average food consumptions on the two diets in both groups are similar and therefore could not be considered an influencing factor.

The data for these groups are summarized in table 1.

Xerophthalmia, pus in the middle ear, in the nose and in the glands of the neck and base of the tongue are among the frequently occurring signs of vitamin A deficiency at the death of the experimental animals. A comparison of the occurrence of these abnormal conditions for all animals showed a somewhat higher incidence on diet II though the difference is probably not significant. On diet I for both groups the percentage incidence of infections was: ears 75.9, eyes 86.0, nose 75.9, tongue 62.0, and on diet II: ears 80.9, eyes 92.0, nose 84.3 and tongue 69.2.

TABLE 1

Comparison of growth, survival and food consumption of rats on diets containing (I) heated and (II) alcohol-extracted casein

| DIET | ANIMALS | | BODY WEIGHT | | | | | | DEPLETION PERIOD, AVERAGE | TOTAL SURVIVAL | | FOOD CONSUMPTION PER DAY |
| | Description | Number used | Initial | | Depletion average | Final | | | | Average | Range | |
			Average	Range		Average	Range					
			gm.	gm.	days	gm.	gm.		days	gm.	days	gm.
I	♂, group A	11	40.5	33–49	98.5	79.7	54–110		28.6	40.0	26–50	7.6
II	♂, group A	11	42.0	33–46	97.0	73.7	64–100		29.3	44.0	33–61	7.3
I	♂, group B	8	45.0	40–52	181.8	131.7	102–150		44.9	65.9	52–87	10.3
II	♂, group B	7	45.3	40–51	179.3	116.6	101–140		44.3	65.0	57–72	10.4
I	♀, group B	9	44.4	37–52	148.5	97.1	72–118		45.1	76.6	64–93	9.3
II	♀, group B	10	42.9	34–51	152.1	98.6	81–118		44.8	76.8	57–98	9.8

From this evidence it is clear that heat-treated casein may be substituted for alcohol-extracted casein in the vitamin A-free basal diet when judged by the growth response, depletion period and total survival time of the experimental animals serving as negative controls.

The animals in group C were used to determine whether the heat-treatment of the casein had changed the nature of the protein or had caused sufficient destruction of essential amino acids to prevent normal growth responses when vitamin A was added as a supplement to the basal diet. If such were

TABLE 2

Comparison of growth and food consumption of vitamin A-depleted rats receiving diets I and II supplemented with cod liver oil

DIETS	RAT NO.	EXPERI-MENTAL PERIOD	TOTAL WEIGHT GAIN	WEEKLY WEIGHT GAIN	FOOD PER DAY	WEIGHT GAIN PER GRAMS FOOD
		days	gm.	gm.	gm.	gm.
I + 3 drops cod liver oil daily	♂ 520	60	232	27.1	18.6	0.208
I + 3 drops cod liver oil daily	♂ 525	60	148	17.3	15.6	0.158
I + 3 drops cod liver oil daily	♀ 523	60	83	9.7	15.7	0.088
I + 3 drops cod liver oil daily	♀ 524	60	67	7.8	15.7	0.071
I + 3 drops cod liver oil daily	♀ 526	60	77	9.1	12.3	0.104
Average for three females			75.7	8.8	14.5	0.087
II + 3 drops cod liver oil daily	♀ 521	60	85	9.9	15.1	0.094
II + 3 drops cod liver oil daily	♀ 522	60	70	8.1	13.3	0.088
II + 3 drops cod liver oil daily	♀ 527	60	73	8.5	14.8	0.082
Average for three females			76.0	8.9	14.4	0.088

the case the use of heat-treated casein might result in misinterpretation of growth responses when making experimental determinations on the vitamin A values of unknown foods or test substances.

The results obtained from group C animals are summarized in table 2. The number of animals used was small but the close agreement of the data from the two diets is believed to justify the conclusion that there was no difference in the two diets. The two males made weight gains which are comparable to those of normally growing rats and the females in both series made average weekly gains of 8.8 gm. and the food consumption was the same in both cases.

SUMMARY

A comparison was made of alcohol-extracted casein and heat-treated casein as a source of protein in the vitamin A-free basal diet of carefully matched experimental rats.

No marked differences were observed in the growth responses, time for depletion or survival period of groups of animals on the two diets, with and without added vitamin A supplement.

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ALLEVIATION OF VITAMIN B DEFICIENCY IN THE RAT BY CERTAIN NATURAL FATS AND SYNTHETIC ESTERS

W. D. SALMON AND J. G. GOODMAN

Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn

Whether fats affect the requirements of animals for vitamin B is very important from the viewpoint of the worker who is investigating the physiological function of this vitamin. That fats decrease the requirement for vitamin B was first reported by Evans and Lepkovsky ('28, '29). Data in support of their initial findings have been submitted by several investigators (Evans and Lepkovsky, '31, '32 a, b, c, d, e, f, '35; Evans, Lepkovsky and Murphy, '34 a, b; Salmon and Guerrant, '30; Salmon and Goodman, '31; Guerrant and Dutcher, '34; Whipple and Church, '36).

Other investigators, however, have reported conflicting or contradictory results (Gregory and Drummond, '32; Kemmerer and Steenbock, '33; Westenbrink, '34 a, b, '35; Sure and Buchanan, '35).

The presentation of further data relating to the problem would, therefore, seem to be pertinent. It is the purpose of this paper to report the most significant of such data which have been obtained in this laboratory and to discuss them in relation to the results obtained in other laboratories.

EXPERIMENTAL MATERIALS AND METHODS

Natural fats and oils. The coconut oil and the hydrogenated cottonseed oil were edible grades obtained from the Capital City Products Co., Columbus, Ohio. Olive oil and cottonseed

oil (Wesson oil) were purchased on the local market. Butter was obtained from the A. P. I. Creamery; it was melted and filtered free from curd and water. The two samples of lard were obtained from the Bureau of Animal Industry Laboratory at Beltsville, Md. The pecan oil was prepared in our laboratory by extracting ground pecan kernels with high test (gas machine) gasoline, removing the solvent, and filtering the oil. Beef fat was likewise prepared by rendering beef suet in an open kettle and filtering.

Prepared esters. The ethyl esters, and glyceryl acetate, propionate, and butyrate were purchased from Eastman. The other glycerides were prepared in our laboratory. The lactic acid was Baker's C. P.; the other acids were the highest grade obtainable from Eastman. The glycerol was Merck's reagent, anhydrous.

A 20% excess of glycerol was used in the esterifications. The temperature varied slightly because of differences in the boiling points of the acids. For the short chain acids the reaction was carried out at atmospheric pressure at a temperature just under the boiling points of the acids. For the longer chain acids the temperature ranged from 170°C., where the reaction usually began, to 240°C. with about a 5-pound reduction in pressure. Agitation of the reaction mixture was accomplished by a stream of dried CO_2 which facilitated removal of the water and prevented oxidation.

When the reaction became impracticably slow the heating was discontinued as it was considered desirable to avoid prolonged heating. The time varied from 5 to 7 hours after the reaction started. After the mixture cooled to 100°C. in an atmosphere of CO_2, 2 gm. of norite and 5 gm. of fuller's earth per 500 gm. original acid were added and after thorough agitation the mixture was filtered. The filtrate was washed with three changes of hot water and again filtered. The acid number was then determined. The lauric, myristic, palmitic and stearic glycerides were dissolved in twice their volume of hot alcohol; the mixture was allowed to cool and as much of the alcohol as possible was drawn off. The alcohol treatment was repeated three times. After the last treatment, the

remaining alcohol was washed from the fat with hot water. The shorter chain esters received an addition of KOH sufficient to neutralize the free acid, the strength of the alkali depending upon the free fatty acid content (Jamieson, '32). The mixture stood at room temperature until the soap separated, after which the soap was removed and the fat was washed three times with water and filtered. The free acid

TABLE 1

Source of acid, saponification number and percentage of acid in esters

ESTER	SOURCE OF ACID [1]	SAPONIFICATION NUMBER	PER CENT ACID
G. acetate	....	771.0	82.5
G. propionate	....	646.0	85.3
G. butyrate	....	557.0	86.8
G. valerate	Butyl cyanide	462.7	84.1
G. caproate	Amyl cyanide	418.9	86.7
Et. caproate	Amyl cyanide	395.0	81.7
G. iso-caproate	....	412.2	85.2
G. heptoate	Heptyl alcohol	372.5	86.4
G. caprylate	Coconut oil	351.1	90.2
G. nonylate	Heptyl bromide and ethyl malonate	317.5	89.5
G. undecylate	Undecylenic acid	281.8	93.5
G. laurate	Coconut oil	263.4	94.0
G. myristate	Nutmeg wax	226.5	92.0
G. palmitate	Animal fat	212.3	96.9
Et. palmitate	Animal fat	201.1	91.8
G. stearate	Animal fat	194.0	98.3
Et. stearate	Animal fat	196.9	94.7
G. oleate	....	187.0	94.1
Et. oleate	....	185.4	93.3
G. lactate	....	469.7	75.2
Coconut oil	....	260.5	93.0

[1] From information furnished by Eastman Kodak Co.

was not neutralized in the glyceryl oleate or lactate nor was the excess glycerol washed out of the lactate. The liquid glycerides were neutral after the refining was completed; the lauric, myristic, palmitic and stearic glycerides contained from 3 to 7% of free acid. The saponification number of all preparations was determined as a basis for calculating the amount of ester to be fed. Table 1 shows the saponification value

and the percentage of acid in the various esters which were used in this study.

Other dietary ingredients. Number 1 casein from Adler and Son, Philadelphia, was used after purification (Salmon and Goodman, '34). The sucrose was commercial refined cane sugar.

The autoclaved yeast was Northwestern powdered yeast.[1] Before being autoclaved it assayed 3–3.3 international units of vitamin B per gram by the pigeon protective method (Salmon, '27). The dry yeast was extracted with gasoline until no more color was removed. The residual gasoline was allowed to evaporate from the yeast which was then spread in layers about 12 mm. deep in enameled pans; the yeast was moistened, autoclaved 8 hours at 120° C., dried at 70° C., ground and stored in tightly closed tinned cans until used.

The brewer's yeast was Vitamin Food Co., no. 1 debittered yeast.[1] It assayed 30 international units of vitamin B per gram by the above method. The vitamin B solid was prepared from an extract of the brewer's yeast by adsorption on fuller's earth at about pH 3.5. It assayed 300 international units per gram.

The diets. Table 2 shows the composition of the diets. In general all the ingredients of the diets were mixed together except the autoclaved yeast, the cod liver oil and the linseed oil; in the diet 55% series, however, the oils and liquid esters were pipetted into the feed jars daily onto weighed portions of the dry mixture of other ingredients. This was necessary to avoid changes in the composition of the diet by settling. The cod liver oil was mixed into diet 40 fat; but in all the other diets both the cod liver oil and the linseed oil were pipetted directly into the feed jars daily. The autoclaved yeast was fed daily in separate jars.

The animals. The rats (Wisconsin strain) were started on test at 23 to 26 days of age at as near 55 gm. average weight

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[1] We wish to express our appreciation to Dr. M. H. Givens of the Northwestern Yeast Company and to President R. M. Allen of the Vitamin Food Company for the yeast used in these studies.

as possible. They were kept in individual cages with raised screen floors, 3 meshes to inch. Feed and distilled water were supplied daily. Weights of rats were recorded weekly.

Digestibility of fat. After the rats had received the fat diets for 1 week, the feces were collected for 4 weeks, except where death of the rats necessitated a shorter collection period. The feces from all of the rats on a given diet were pooled and dried at 45°C. They were then weighed and aliquots (or all the feces from a group if the amount permitted) placed in Soxhlets. They were extracted with ether for 24 hours, then

TABLE 2

Percentage composition of basal diets

INGREDIENTS	DIET						
	Low fat	High fat	40 fat	5%[2]	23%[2]	36%[2]	55%[2]
Purified casein	18.0	32.0	22.5	18.0	18.0	22.5	32.0[3]
Salt 186[1]	4.0	7.0	5.7	4.0	4.0	5.7	7.0
Agar	1.0	1.8	1.0	1.0	1.0	1.0	1.8
Fat or ester	....	59.2	40.0	5.0[4]	23.0[4]	36.0[4]	55.0[4]
Sucrose	77.0	....	28.8	....[5]	....[5]	....[5]	

[1] J. Biol. Chem., 1930, vol. 89, p. 199.

[2] Indicates fatty acid equivalent of ester or fat in diet.

[3] In the case of glyceryl acetate it was necessary to reduce the casein to 28, salt to 5, agar to 1% in order to get the equivalent of 55% acetic acid in diet.

[4] These figures represent the fatty acid equivalent of the esters; the percentage of ester was necessarily affected by the molecular weight of the fatty acid.

[5] Sucrose was added to make 100%.

made acid to Congo red with a few drops of concentrated H_2SO_4 and again extracted with ether for 12 hours. After the ether was dissipated the two fractions of extract were weighed. From these weights and the amount of fat ingested during the period the percentage of fat retained was calculated.

Preparation and examination of tissue fats. The fatty acids from livers and brains of several groups of rats were used for magneto-optic (Allison and Murphy, '30) and refractive index determinations. These rats were killed by decapitation; the livers and brains were removed as rapidly as possible and dissolved in 10 ml. of boiling 60% KOH. The solutions were

heated in covered flasks for 4 hours on a water bath. When cool, each sample was made to 100 ml. and 1 ml. removed and diluted for magneto-optic study. The remainder was acidified with HCl and shaken with three 25-ml. portions of ether. The ethereal solutions were washed with water and the three fractions combined. After the ether had dissipated, the dry material was extracted with three 10-ml. portions of ether which were filtered through dry fat-free filter paper. The filtrates were evaporated to dryness. The residue, designated as the fatty acid fraction, was weighed and the refractive index was determined at 60°C. with an Abbé refractometer.

RESULTS

Effect of coconut fat. The approach to the problem under consideration was to determine whether the addition of various fats to a vitamin B-free diet would increase the gain in weight or the time required for rats to develop beriberi. Beriberi is used throughout this paper for the onset of symptoms which appear in the late stages of vitamin B deficiency. It is characterized by varying degrees of anorexia, marked incoordination or muscular weakness, and finally by prostration and death within a few days unless treatment is given. Spasticity almost invariably appears in the animals which develop beriberi on diets containing moderate amounts of fat; it sometimes is not observed in animals receiving a vitamin B deficient diet which is low in fat, in which cases the picture may be one of inanition and general weakness.

The weight curves in figure 1 show that the diet containing 23% of coconut oil produced a slightly larger initial gain than the low fat diet but did not delay the onset of beriberi. A diet containing 59.2% of the oil, however, not only increased the gain significantly but had a pronounced effect in inhibiting the symptoms of beriberi. Of the six rats started on this diet, only one developed beriberi and that was after 26 weeks. This was more than four times as long as was required by the rats receiving the low fat diet or the diet containing only 23% of coconut fat. The experiment was continued for 48 weeks

without another case of beriberi developing. These rats received only 250 mg. of autoclaved yeast per rat daily which was the same amount received by those on the low fat and the 23% fat diets. Three other groups of rats were fed on the 59.2% fat diet to determine the effect of increasing the autoclaved yeast to 500 mg. daily as well as the effect of adding vitamin B. The weight curves for these are also shown in
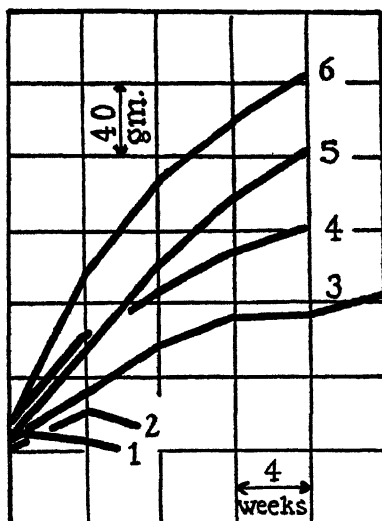
Fig. 1 Composite growth curves of rats. Curve 1, low fat diet with 250 mg. autoclaved yeast; curve 2, 23% coconut oil with 250 mg. autoclaved yeast; curve 3, high fat (59.2% coconut oil) with 250 mg. autoclaved yeast; curve 4, high fat with 500 mg. autoclaved yeast; curve 5, high fat with 250 mg. autoclaved yeast and 10 mg. vitamin B solid; curve 6, high fat with 500 mg. autoclaved yeast and 10 mg. vitamin B solid per rat daily. The ends of curves 1 and 2 indicate death of the last rat in group. The rats represented by curve 3 were continued for a total of 48 weeks with but one case of beriberi. Those represented by curves 4, 5 and 6 were discontinued after 16 weeks. Curve 6 represents two rats; other curves, six rats each.

figure 1.   Although increasing the autoclaved yeast from 250 mg. to 500 mg. per day improved the rate of growth, the improvement was less than that resulting from the addition of 10 mg. of vitamin B solid to the 250 mg. of autoclaved yeast. Moreover, there was a further stimulation of the growth rate

by adding 10 mg. of vitamin B solid to the 500 mg. level of autoclaved yeast.

It is thus apparent that the high fat diet is improved by the addition of vitamin B or some other heat labile factor carried by the solid. It also appears that at the 250-mg. level of autoclaved yeast the limitation of growth is more dependent upon a lack of this factor than of vitamin G.

Effect of different levels of autoclaved yeast. That increasing the level of autoclaved yeast increased the growth produced by the high fat diet was shown in figure 1. Further data in table 3 show this more strikingly. The effect of the increased level of autoclaved yeast was much greater with the

TABLE 3

Average gain in weight and time to onset of beriberi in rats receiving different levels of autoclaved yeast[1]

DIET	YEAST DAILY	NUMBER OF RATS	AVERAGE TOTAL GAIN	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET	LENGTH OF TEST
	mg.		*gm.*	*%*	*days*	*days*
High fat[2]	250	4	61	25	107	127
High fat[2]	500	4	128	0	...	127
High fat[2]	1000	4	164	0	...	127
Low fat	250	6	2	100	37	45
Low fat	500	6	6	83	59	119

[1] Each rat received 0.10 ml. cod liver oil per day.
[2] Coconut oil was used in the high fat diet in these experiments.

high fat diet than with the low fat diet. Even with the latter there was a considerable delay in the onset of beriberi. Our interpretation of these results is that the autoclaved yeast still contained traces of vitamin B. Since the requirement of the rat for vitamin B is small, the effects of such traces become apparent when large amounts of autoclaved yeast are fed. The effects are emphasized by the peculiar action of fat.

Comparison of different fats. A series of preliminary experiments was conducted to determine the best level of fat for comparing the effects of fats when added to a vitamin B free diet. On the basis of these tests a level of 40% of the test fat in the diet was selected. Table 4 includes the results of this comparison.

Coconut oil was the most effective of the natural fats both in producing gain in weight and in delaying the onset of beriberi. Cottonseed oil and pecan oil ranked next in delaying the development of beriberi but did not produce as much growth as lard or butter. The latter ranked at the bottom of the list in its ability to delay the occurrence of beriberi but was next to coconut oil in its effect on growth. It is probable that palatability of the fat may affect growth more than it does the

TABLE 4 [1]

Average gain in weight and time to onset of beriberi in rats receiving vitamin B deficient diet (40 fat) containing various fats at a level of 40% [2]

KIND OF FAT	NUMBER OF RATS	AVERAGE TOTAL GAIN	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET
		gm.	%	*days*
Myristin 30 } Hard lard 10 }	6	47	0	.. [3]
Coconut	6	28	83	60
Cottonseed	11	6	100	51
Pecan	6	9	100	46
Hyd. cottonseed	24	12	95	40
Linseed	8	5	100	40
Hard lard	18	20	83	40
Soft lard	6	17	100	37
Hard lard 30 } Myristin 10 }	6	17	100	37
Beef	11	5	100	35
Olive	6	14	100	35
Butter	7	21	100	34

[1] The data in this table were taken from a master's thesis submitted to this institution by J. G. Goodman.

[2] Each rat received 250 mg. autoclaved yeast and 0.10 ml. cod liver oil per day.

[3] No beriberi at end of 102 days.

time required for the onset beriberi. The differences in the natural fats with the exception of coconut and cottonseed oil do not seem to be important. The effect of a mixture of 3 parts of myristin and 1 part of lard, however, is very striking.

Comparison of various esters. A series of tests on synthetic esters of various fatty acids was next undertaken. Most of these esters were fed at a level equivalent to 23% of fatty acid in the diet. Glyceryl caprylate was also fed at the equivalent of 5, 36 and 55% and glyceryl caproate at the equivalent

of 5% fatty acid. The essential unsaturated fatty acids were supplied by 0.20 ml. of U. S. P. linseed oil per rat daily. All rats in this series were subjected to a preliminary, 2-week depletion period on the low fat diet in an attempt to reduce their body stores of vitamin B to a low level before the feeding of the ester-containing diets was begun. It was expected that all rats receiving diets containing only 23% of the esters would succumb to the deficiency and that the time of onset of premortal symptoms as well as the amount of growth could be used in evaluating the esters. As the experiment progressed it became apparent that some of the esters might prolong life indefinitely. Consequently the feeding of the esters was discontinued after 12 weeks in all groups that had not developed beriberi previous to that time.

Table 5 presents the results of this series. At the 23% level glyceryl caprylate produced significantly more growth than any of the other esters. Glyceryl caproate ranked next, the iso-caproate apparently being as good as the normal. Ethyl caproate produced only slightly less growth than the glyceryl ester. It is interesting that glyceryl heptoate produced nearly as much growth as the caproate; the heptoate gave about the same growth as the laurate and slightly better than the myristate which did not produce significantly more gain in weight than the nonylate and the undecylate. The only cases of beriberi which occurred at the 23% level were on ethyl palmitate, glyceryl palmitate, stearate, and acetate, and coconut fat which was included in the series as a control. Of eighteen rats on the low fat diet as controls, fourteen developed beriberi in an average of 43 days. As compared with this the palmitate, stearate, and acetate actually decreased the time required for the onset of beriberi. The acetate stimulated growth slightly but the palmitate and stearate produced digestive disturbances and a steady loss of weight. The butyrate also decreased the survival period but the symptoms suggested a toxic effect rather than vitamin B deficiency. Later tests showed a similar toxic action in the presence of an adequate vitamin B intake.

TABLE 5

Average gain in weight and time to onset of beriberi in rats receiving vitamin B deficient diets[1] containing various glyceryl and ethyl esters

DIET	ACID EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN 6 WEEKS[4]	AVERAGE GAIN 12 WEEKS[4]	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET[4]	ESTER RETAINED
Series A[2]	%		gm.	gm.	%	days	%
G. caprylate	55	4	57	Discontinued	0	..	99.2
G. caprylate	36	4	51	Discontinued	0	..	....
G. caprylate	23	4	57	101	0	..	98.4
G. caprylate	5	4	47	75	0	..	....
G. caproate	23	4	46	72	0	..	99.0
G. iso-caproate	23	4	46	Discontinued	0	..	98.7
Et. caproate	23	4	42	64	0	..	....
G. heptoate	23	4	38	64	0	..	98.0
G. laurate	23	4	31	63	0	..	97.4
G. myristate	23	4	28	47	0	..	96.3
G. nonylate	23	4	23	42	0	..	98.4
G. undecylate	23	4	20	40	0	..	98.8
Et. oleate	23	4	23	34	0	..	96.9
G. propionate	23	4	19	33	0	..[5]	98.9
G. lactate	23	4	17	27	0	..	94.6
G. oleate	23	4	15	25	0	..	97.4
G. caproate	5	4	12	32	0	..	....
G. valerate	23	4	17	17	0	..[6]	98.7
Low fat diet	..	18	7[7]	Died	77	43	93.5
Coconut	23	8	13[7]	Died	62	40	97.6
Et. palmitate	23	4	0[8]	Died	75	38	92.6
G. acetate	23	4	24[7]	Died	100	32	99.0
G. palmitate	23	4	0[8]	Died	100	30	91.5
G. stearate	23	4	0[8]	Died	100	17	74.4
G. butyrate	23	4	0[8]	Died	...	20[9]	97.6
Series B[3]							
G. caprylate	5	4	10	11	25	63	....
Low fat diet	..	14	0[8]	Died	100	25	....

[1] Each rat received 0.10 ml. cod liver oil and 0.20 linseed oil per day.

[2] Each rat in series A received 500 mg. autoclaved yeast per day.

[3] Each rat in series B received 250 mg. autoclaved yeast per day.

[4] All rats received the low fat diet for a 2-week depletion period; the time of experiment was reckoned from the end of this period.

[5] One rat died 3 days after starting on glyceryl propionate.

[6] One rat died 3 days after starting on glyceryl valerate.

[7] Average gain to maximum weight.

[8] These rats lost weight from start of ester-containing diets.

[9] Average length of life; symptoms not typical of beriberi.

Glyceryl caprylate was also fed at levels corresponding to 5, 36 and 55% of caprylic acid in the diet. The growth at 6 weeks was no better on the higher percentages than on the 23% level. On 5% of glyceryl caprylate the growth was about 17% less at 6 weeks and 25% less at 12 weeks than on 23%. As at the 23% level, glyceryl caproate at the 5% level was less effective in supporting growth than the caprylate.

When the dosage of autoclaved yeast was reduced to 250 mg. per rat daily, glyceryl caprylate at the 5% level did not entirely prevent beriberi, although only one rat in a group of four went down in the 12-week test period. In contrast with this, fourteen rats on the low fat diet and likewise receiving 250 mg. of autoclaved yeast showed a 100% incidence of beri-beri; the average time required was 25 days after the beginning of the test period.

In view of the belief of some investigators that vitamin B may function in the metabolism of lactic acid, it is interesting to note the effect of this acid. None of the rats receiving glyceryl lactate equivalent to 23% of lactic acid in the diet, developed beriberi in the 12-week test period. Either less vitamin B was required in the metabolism of lactic acid than of sucrose or the c. p. lactic acid carried traces of vitamin B as an impurity.

The percentage of food fat retained was high and apparently too uniform to account for the differences in efficiency of the esters. The only marked reductions in the retention were with the esters of palmitic and stearic acids. There were always evidences of digestive disturbances when the esters of these acids were fed. It is possible that unabsorbed residues of these substances which melt above the body temperatures are irritating to the intestinal mucosa. The apparent poor retention of food fat with the low fat diet is probably due to the excretion of fatty material through the intestinal tract. This would obviously cause a greater error with a low fat diet than with a moderate or high fat diet.

Curative tests with glyceryl caprylate and caproate. Since some of the esters in the preceding tests were so potent in

preventing beriberi, it was decided to determine what would be the effect of feeding them to rats which had developed the disease on other diets.

The curative action of diets containing 23% of glyceryl caproate or caprylate was very striking. The rats whose weight curves are shown in figure 2 were in the spastic stage
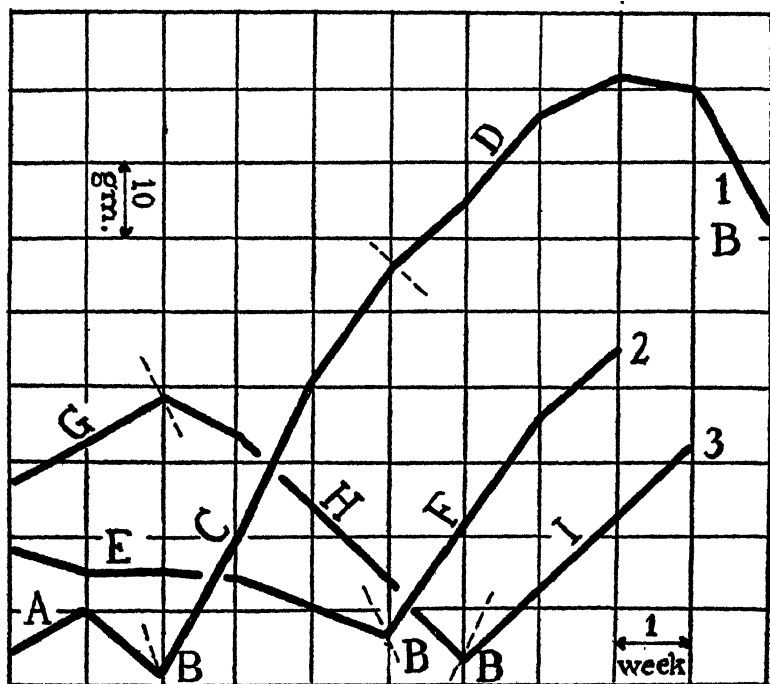
Fig. 2 Composite growth curves of rats. Curve 1: A, 23% glyceryl acetate; C, 23% glyceryl caprylate; D, 23% glyceryl acetate. Curve 2: E, low fat; F, 23% glyceryl caproate. Curve 3: G, 23% glyceryl iso-caproate; H, low fat; I, 23% glyceryl iso-caproate. B indicates onset of beriberi. Curve 1 represents one rat; curve 2, two rats; curve 3, three rats.

.of beriberi. It was necessary to turn the feed jars on the side and support the rats for their first feeding of the ester-containing diets. The cures were nearly as rapid as when brewer's yeast was fed. These curative tests apparently show that to a certain extent there may be an actual substitution of fatty acids for vitamin B and carbohydrate in the

metabolism of the rat. One curve in figure 2 is particularly interesting: it shows that when 23% of acetic acid as the glyceride was fed, beriberi developed and was cured by substituting caprylic for acetic ester but again developed when the feeding of the acetic ester was resumed. Numerous other cures were effected, the results indicating a correlation between the curative and the protective efficiency of the esters.

Growth produced by various esters and natural fats when vitamin B intake was adequate. It was evident from the previous tests that esters and natural fats varied significantly in preventing beriberi and supporting growth on diets that were free or nearly free of vitamin B. These results suggested the question whether differences in efficacy were due to differences in the general nutritive value of these substances which would be apparent even when the diet contained adequate vitamin B. An experiment was initiated to answer this question.

For this purpose it seemed necessary to use a diet in which the sole non-nitrogenous source of energy would be the fat to be tested. With adequate vitamin B, any carbohydrate in the diet would be utilized for growth and would tend to obscure the true value of the fat. The diets in this series contained fat or synthetic ester equivalent to 55% of fatty acid. However, some of the substances which were injurious at this high level were also tested at a level equivalent to 23% of fatty acid.

The results are shown in table 6. When vitamin B was supplied, coconut oil, olive oil and lard were of practically identical values as sources of energy. Moreover none of these were significantly superior to sucrose, at least within the 6-week test period. Beef fat, cottonseed oil and butter fat were slightly less valuable than the above. Glyceryl caproate or laurate were distinctly inferior to any of the natural fats or to sucrose.

A mixture of equal parts of glyceryl heptoate and nonylate gave poor results, two of the rats dying before the end of the test period. Two rats died on the glyceryl acetate diet also.

Glyceryl palmitate was even more harmful; the average life of the four rats on this diet was only 8 days.

Glyceryl acetate equivalent to 23% acetic acid lowered the nutritive value of the diet markedly but less than glyceryl or ethyl stearate at this level. Glyceryl butyrate was distinctly harmful, only one rat of four living 6 weeks.

TABLE 6

Average gain in weight and average food of rats receiving adequate vitamin B and diets containing various esters or natural fats [1]

ESTER OR FAT	ACID EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN 6 WEEKS	AVERAGE FOOD 6 WEEKS	ESTER RETAINED
	%		gm.	gm.	%
Coconut	55	4	130	248	98.8
Olive	55	4	128	248	97.7
Lard	55	4	126	274	96.1
Low fat	55	4	124	391	89.8
Beef	55	4	114	253	94.9
Cottonseed	55	4	107	220	96.6
Butter	55	4	105	226	96.9
G. caproate	55	4	84	224	99.4
G. laurate	55	4	67	170	97.9
G. heptylate nonylate	55	4	65[2]	176	99.2
G. acetate	55	5	45[2]	216	87.2
G. palmitate	55	4	..[3]	...	....
G. stearate	23	4	54	295	19.1
Et. stearate	23	4	37	251	48.9
G. butyrate	23	4	—22[4]	...	98.2
G. acetate	23	4	90	349	99.3

[1] Each rat received 500 mg. brewer's yeast, 0.10 ml. cod liver oil, and 0.20 ml. linseed oil per day.

[2] Two rats died before end of 6-week period.

[3] Average life was 8 days on this diet.

[4] Only one rat lived 6 weeks on this diet.

These results show that fats do not rank the same when the diet contains adequate vitamin B as when it is deficient in vitamin B. Differences in their effects on rats receiving vitamin B deficient diets are not due to differences in their general nutritive value.

Effect of vitamin B deficiency on fats of brains and livers. We next tried to determine whether vitamin B or fat in the diet affects the nature of the fatty acids in the tissues of rats. Several groups of rats received controlled amounts of food and after suitable intervals their brains and livers were prepared for study as described under methods. Weights and refractive indexes of the fatty acid fraction from these tissues were determined and are shown in table 7.

TABLE 7

Average weights and refractive indexes (at 60°C.) of fatty acid fraction from brains and livers [1]

GROUP	NUMBER OF RATS	DIET	VITAMIN B [2]	AVERAGE WEIGHT OF RAT	BRAIN FATTY ACID		LIVER FATTY ACID	
					Average weight	Ref. ind.	Average weight	Ref. ind.
				gm.	mg.		mg.	
1	3	Low fat	—	73	84	1.4714	96	1.4644
2	3	Low fat	+	100	87	1.4680	173	1.4569
3	3	High fat [3]	—	80	47	1.4713	70	1.4670
4	3	High fat [3]	+	100	75	1.4709	213	1.4606
5	3	Low fat	—	43	88	1.4703	63	1.4618
6	2	Low fat	+	43	57	1.4750	54	1.4624
7	5	High fat [3]	—	48	60	1.4707	70	1.4629
8	3	High fat [3]	+	47	65	1.4718	70	1.4652
9	3	Low fat	+	198	101	1.4705	246	1.4535
10	4	Stock	+	64	57	1.4689	71	1.4626

[1] Each rat in groups 1 to 8, inclusive, received 0.10 ml. cod liver oil, 0.20 ml. linseed oil, and 250 mg. autoclaved yeast per day.

[2] Each rat in groups 2, 4, 6, 8 received 20 mg. vitamin B solid on alternate days; each rat in group 9 received 500 mg. brewer's yeast per day and the usual dosage of linseed and cod liver oils.

[3] Wesson oil was used in the high fat diet in this series.

The rats in groups 1 to 4, inclusive, were started on experiment simultaneously. All rats received vitamin B for 4 weeks. Groups 2 and 4 were then killed. The vitamin B was omitted and groups 1 and 3 were continued on their respective diets for 6 weeks. At this time all the rats in group 1 were losing weight and one rat was showing spastic symptoms of beriberi. The rats in group 3 were likewise losing weight due to limitations of their food since the energy intake was kept uniform for all four groups in this experiment.

The weights of the fatty acid fractions of both brains and livers were less in groups 1 and 3 which did not receive vitamin B than in groups 2 and 4 which received this vitamin. The most surprising feature, however, is the smaller weight of the fatty acid fraction of these tissues in group 3 which received the high fat diet without vitamin B than in group 1 which received the low fat diet without vitamin B. The refractive indexes were somewhat higher in the groups which did not receive vitamin B, the fat from the livers showing more difference than the fat from the brains.

The energy intake in groups 5, 6, 7 and 8 was likewise kept uniform but it was more rigidly limited than in the preceding groups. The maximum daily intake was kept at 12 Cal. per rat. As the deficient rats reduced their consumption below this amount, the diet of the rats receiving vitamin B was correspondingly reduced. It was found impossible to reduce the energy intake of the rats that received vitamin B to the low levels reached by the deficient rats in the incipient stages of beriberi; if this was done the rats receiving vitamin B always died before the ones not receiving vitamin B. Hence, 10.8 Cal. per rat daily was adopted as the minimum level for those which received vitamin B. It is interesting to note that group 6 receiving the low fat diet and vitamin B actually had less fatty acid fraction in both brains and livers than group 5 which received the same diet without vitamin B. The refractive indexes were also slightly higher for the group which received the vitamin. These relationships were reversed from those in groups 1 and 2 where the energy allowance was more liberal. The weights of the fatty acid fraction in groups 7 and 8 were not so divergent but the refractive indexes showed the same reverse relationship.

Group 9 received the low fat diet, ad libitum, and vitamin B for 10 weeks. Group 10 was a group of rats from the stock colony; they were just a few days older than the rats in the other groups were when placed on experiment. The refractive indexes in these two groups were not markedly different from those of the other groups, except they were quite low in the fatty acid fraction from the livers in group 9.

On the whole it does not appear that a deficiency of vitamin B had any direct influence on the refractive index of the fatty acid fraction from the brains or livers.

Data from the magneto-optic studies will not be presented in this paper. In groups 1, 2, 3 and 4 there was a lower content of caproic and caprylic acids in the brains and livers of the deficient rats than of those receiving vitamin B. In groups 5, 6, 7 and 8 this was reversed. In all cases the content of these acids was very low and the differences did not appear to be significant.

The magneto-optic minima which have been reported for formaldehyde (Sommer, '33) and formic acid (McGhee and Laurenz, '33) were observed in preparations from the brain, liver and blood of rats in all groups. The circle readings indicated a higher concentration of these compounds in the tissues of rats receiving the low fat diet than in those receiving the high fat diet; they also indicated a higher concentration of formic acid in the tissues of rats receiving vitamin B than in those of rats not receiving this factor. Although the existence of formic acid in animal tissues has been reported, we do not believe the presence of formaldehyde has been previously observed. If its presence can be confirmed by other methods, some very interesting possibilities regarding its role in intermediary metabolism will need investigation.

DISCUSSION

In comparing our results with those of other investigators it is well to keep in mind the possible influence of certain differences.

In the experiments of Evans and Lepkovsky and in our early experiments with natural fats no provision was made for a supply of essential unsaturated fatty acids other than that furnished by the fat being tested for its vitamin B-sparing action. This may have been a limiting factor in certain groups where low percentages of fats were fed. Where saturated fatty acid esters are the chief source of energy, the necessity for the inclusion of unsaturated fatty acids containing more than one double bond has been indicated (Evans

and Lepkovsky, '32 e and f). These essential unsaturated acids were adequately provided in all of our later experiments by the feeding of 0.20 ml. of U. S. P. linseed oil per rat daily (tables 5, 6, 7, and fig. 2).

Also in our experiments the autoclaved yeast was weighed separately and fed in uniform daily dosage to all the rats in a comparison series. Therefore, variations in food consumption did not result in variations in the intake of autoclaved yeast. The early work in our laboratory indicated that considerable variation in the results could be produced by varying the level of autoclaved yeast (Salmon and Guerrant, '30). This has been confirmed by the more recent work of Evans and Lepkovsky ('34 a) and by the results reported in this paper. The increase in growth and in the time required for onset of beriberi which results from increasing the level of autoclaved yeast, has been attributed to an increase of vitamin G by Evans, Lepkovsky and Murphy ('34 a). We are not inclined to consider this an adequate explanation of the effect. Our data in figure 1 show that adding 10 mg. of vitamin B solid was more effective than increasing the autoclaved yeast from 250 mg. to 500 mg. Moreover, adding 10 mg. of vitamin B solid to the 500 mg. allowance of autoclaved yeast again increased the rate of growth. It is evident that there was a marked stimulation of growth by some heat labile factor carried by the vitamin B solid. It is again shown in table 5 that increasing the level of autoclaved yeast from 250 mg. to 500 mg. when the low fat diet was being fed, prolonged the time required for the development of beriberi an average of 18 days. The effect is, therefore, not limited to a fat containing diet.

These results seem to us to indicate that the autoclaved yeast still contains traces of vitamin B. The effect of such traces is apparent even when a low fat diet is being fed but is accentuated by a high fat diet.

The data in this paper do not lend support to the view that a high protein intake is essential for the alleviation of vitamin B deficiency by fat. In the diets containing 40% or more of

fat, the protein content of the diets was increased in proportion to the increase in energy resulting from the substitution of fat for carbohydrate. In the diets containing esters equivalent to 23% of fatty acid, the casein was kept constant at 18%. The results reported in table 5 show that with this normal level of protein the effect of the esters was very pronounced.

The efficiency of an ester of the fatty acids in alleviating vitamin B deficiency in the rat appears to be related to the length of the carbon chain of the acid which the ester contains. In our experiments the efficiency was maximum at the 8-carbon acid. It decreased in each direction from this point until acetic on the one end and palmitic and stearic on the other end of the series were not effective at the 23% level. In fact the onset of beriberi was hastened by the replacement of sucrose with these acids. The odd carbon acids were slightly irregular but they followed the same trend as the even carbon acids.

Only in the results from the esters of palmitic and stearic acids was there evidence that decrease in efficiency might be attributed in part to poor absorption of the long chain compounds. The effectiveness of other members of the series as measured by the amount of growth produced, began to decrease, however, before there was any appreciable decrease in absorption. Moreover, oleic glyceride which was absorbed as well as lauric and myristic glycerides produce much less growth than either of these. Also acetic acid which was almost completely absorbed ranked very low.

The data on absorption or retention of the food fat indicate no impairment of fat digestion as a result of vitamin B deficiency. In the light of this it would seem that the reduction in pancreatic lipase activity in vitamin B deficient rats reported by Sure, Kik and Buchanan ('35) is not significant.

Despite differences in methods our results agree quite well with the major findings of Evans and Lepkovsky. We have studied a greater number of acids and have found a closer relationship between the length of the carbon chain of the

saturated acids and their effectiveness. Our data do not support the conclusions of the above investigators that the solid fats are more effective than the liquid fats. Furthermore, our results show that a high intake of vitamin G (autoclaved yeast) and of protein is not requisite to the demonstration of the improvement of vitamin B deficient diets by esters of certain fatty acids.

The results of Kemmerer and Steenbock ('33) do not necessarily conflict with the above results. It is evident that the fat diets which have been used do not completely obviate the necessity for vitamin B. It seems reasonable to expect that rats would first deplete their available body stores of vitamin B and then depend upon fatty acids to meet the nutritive emergency. In our experiments the esters of caproic and caprylic acids apparently cured cases of spastic beriberi. Presumably the usable stores of vitamin B were exhausted and fatty acid appeared to have substituted for this factor. This indicates that conservation of previously stored vitamin B is not an essential part of the action of fatty acids in alleviating vitamin B deficiency in rats.

The action of fats and the different effects of the fatty acids in conditions of vitamin B deficiency in the rat, present a puzzling problem. The advantage of a high fat diet over a low fat diet seems to disappear when the diet contains adequate vitamin B and the essential unsaturated fatty acids. Moreover, the ranking of the esters of certain fatty acids over the natural fats, when the diet is deficient in vitamin B, is reversed when a sufficiency of this factor is supplied. It thus appears that the 'vitamin B sparing action' of these substances is not due to any superior general nutritive value of certain fatty acids but is in some way specifically related to the deficiency.

The suggestion of Whipple and Church ('36) that vitamin B plays a role in the synthesis of fat from carbohydrate is a possibility to be considered. However, Schrader ('34) has shown in this laboratory that vitamin B deficient rats receiving a carbohydrate diet have respiratory quotients averaging 1.26.

Although this falls short of conclusive proof of transformation of carbohydrate into fat, it suggests that such a change is being made. Furthermore, if the sole function of vitamin B were as a catalyst in the synthesis of fat, it should be possible to compose a diet which would meet the requirements of animals for fat and support normal growth without the presence of vitamin B. We have no evidence that this can be done.

The idea that vitamin B functions in the metabolism of carbohydrate has had numerous proponents. The beneficial effect of replacing carbohydrate with fat in a diet which is inadequate in vitamin B, tends to support this idea. However, no one has yet been able to locate definitely the stage in the intermediary metabolism which is dependent upon this factor. Our data contribute to this only in showing that a relatively large amount of lactic acid ester in the diet tends to delay rather than hasten the onset of beriberi in the rat. This may be interpreted as indicating that vitamin B is not specifically related to the degredation of lactic acid.

SUMMARY

High percentages of fat in vitamin B deficient diets increased the rate of growth and decreased the incidence of beriberi in rats. Coconut fat was the most effective natural fat tested.

The effectiveness of esters of single fatty acids in alleviating the symptoms of vitamin B deficiency in rats depended upon the length of the carbon chain of the fatty acid. The effectiveness was maximum at the 8-carbon acid and decreased in each direction from this point.

Spastic beriberi in rats was cured by the feeding of glyceryl caprylate or caproate.

The apparent nutritive value of fats and single acid esters in vitamin B deficient diets was not the same as in diets that contained adequate vitamin B.

Increasing the autoclaved yeast intake improved the rate of gain and delayed the onset of beriberi in rats receiving vitamin B deficient diets but the addition of a source of vitamin B was more effective than increasing the autoclaved yeast.

A high intake of protein or of vitamin G (autoclaved yeast) was not a requisite for the action of fat in alleviating the effects of a vitamin B deficient diet.

Determinations of the refractive index of the fatty acid fraction of fat from the brains and livers as well as magneto-optic determinations of caprylic and caproic acids from these fractions failed to show any differences that could be attributed to the effect of vitamin B deficiency.

The magneto-optic minima of formaldehyde and formic acid were found in the brains, livers and blood of rats used in this study.

The results are discussed in relation to those obtained in other laboratories.

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THE RELATION OF INGESTED CARBOHYDRATE TO THE TYPE AND AMOUNT OF BLOOD AND URINE SUGAR AND TO THE INCIDENCE OF CATARACT IN RATS [1]

HELEN S. MITCHELL, OREANA A. MERRIAM AND GLADYS M. COOK

Massachusetts State College, Amherst

The discovery and repeated confirmation of the fact that lactose and galactose rations may cause cataract in rats has been followed in this laboratory by extensive investigations as to the nature of the metabolic disturbances involved. No gross tissue pathology other than that of the lens is evident in these animals and a few histological studies of the endocrine organs have failed to reveal any significant changes. Numerous workers have found abnormal blood and urine sugar levels after the ingestion of large amounts of lactose or galactose both in man and in animals. Cori and Cori ('28) explained the more severe galactosuria after the ingestion of galactose on the ground of more rapid absorption taking place when the delay due to hydrolysis was not involved. Harding et al. ('34) concluded that there is, proportionally, a much larger excretion of ingested galactose in the urine in the rat than in man, and that there appears to be a lower rate of conversion of galactose into its metabolites in the rat. The present studies into the nature of the blood and urine sugar of rats fed high levels of these two sugars have been made in connection with further observations on factors influencing the rate of development of lens opacities.

Although high blood sugar values in rats rendered cataractous by lactose and galactose rations have been found repeatedly in this laboratory, and confirmed by Day ('36), several pertinent questions still remain to be answered. How much of this abnormally high blood sugar is glucose and how much is galactose? Does the speed of cataract development correlate with the degree of galactosemia? How may other combinations of carbohydrates affect the blood sugar level and the crystalline lens?

<center>EXPERIMENTAL PROCEDURE</center>

In the present study the general procedure and experimental rations used were similar to those previously reported by Mitchell and Dodge ('35) and by Mitchell ('35). The skeleton ration formula in which the various carbohydrates were substituted was as follows:

	%
Carbohydrate	70
Casein	15
Salt mixture (O. and M.)	4
Crisco	9
Cod liver oil	2

Records of body weight, food intake, and cataract development have been kept systematically. Studies of blood and urine have been made on rats fed on rations containing 62 and 70% lactose, 25 and 35% galactose, 35% fructose, 35% xylose and 70% starch. Some preliminary observations have been made with Insulin-protamine injection in rats on the cataract producing 25% galactose ration. Four strains of rats have been studied on various cataract producing rations in an effort to determine whether differences in cataract susceptibility reported by Mitchell ('36) might be correlated with differences in blood or urine sugar levels. The rats used routinely were from the Battle Creek (B.C.) colony and the other strains studied were from the Johns Hopkins (J.H.), the Wistar (W.) and the local colonies (M.S.C.).

Blood sugar. Blood samples of 0.1 cc. (or 0.05 cc.) were drawn from the tail and blood sugar determinations made by

the Benedict micro-colorimetric method.[2] A free flow of blood was obtained by warming the tail in warm water before bleeding. There were practical difficulties in attempting to take blood samples from very young rats, but it was desirable to study the blood sugar during the period of cataract development if possible. Since cataracts developed in rats after 10 to 20 days on galactose rations (between the fortieth and fiftieth day of age) it was not always possible to obtain a long series of blood sugar determinations before the cataract developed. In the case of rats on lactose rations, lens changes were more delayed and there was no difficulty in obtaining a series of blood samples during the period of cataract development.

Blood sugar determinations were made under as nearly standard conditions as possible. A known amount of food (usually 3 gm.) was given early in the morning following a 16-hour fast and left in the cage for 1 hour. Usually the entire amount of food was eaten and most of it during the early part of the hour. Blood samples were taken $\frac{1}{2}$ hour later presumably when the blood sugar level would be close to the maximum. Day ('36) found the peak somewhere between 1 and $2\frac{1}{2}$ hours.

Various devices for differentiating glucose and galactose in blood have been investigated but the simplest and most satisfactory for routine procedure seemed to be a yeast fermentation. The Benedict micro-colorimetric method provided sufficient supernatant fluid after precipitation of proteins for the determination of the total sugar, leaving a remainder of slightly less than 3 cc. for yeast fermentation. Preliminary tests showed that glucose in the concentrations encountered in blood filtrates completely fermented in 30 to 40 minutes, while only a trace of added galactose was lost in that time. The fermentation procedure was standardized as follows: 2 cc. of a 10% baker's yeast suspension was centrifuged for 15 minutes, the supernatant fluid discarded, and the tube

wiped as dry as possible with a cotton swab. The 3 cc. of solution to be fermented was then mixed with the yeast cells in the centrifuge tube and allowed to stand at room temperature for 40 minutes with occasional stirring. After centrifuging, the second sugar determination was made upon the supernatant fluid. The reducing sugar present after fermentation was assumed to be galactose since control tubes containing glucose gave negative results and no other slowly fermenting sugar could logically be present in the blood of rats fed on starch and galactose rations.[3]

Attention is called to the fact that this fermentation method is only approximate, since the moisture retained by the yeast cells necessarily dilutes the solution to be fermented and, consequently, there is always a slight reduction in the sugar concentration due to this dilution irrespective of any fermentation, but no correction has been made to compensate for it. More prolonged centrifuging to pack the yeast cells harder and reduce the moisture content tended to reduce this error in the more recent determinations.

Urinary sugar. Determinations of total and nonfermentable sugar were made on 24-hour urine specimens collected in metabolism cages designed for the purpose. Most satisfactory specimens were obtained when the rats were fasted during the collection period. When food was allowed in the metabolism cage the danger of food contamination was present in spite of every precaution. The volume of urine from very young rats was so small as to involve a large error due to evaporation and accumulation on the sides of the cages and funnels. Nevertheless, an indication of the type of urinary sugar was determined when the volume obtained was sufficient for the fermentation procedure. Since cataracts developed so early on the galactose rations, it was not expedient

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[3] Galactose was obtained from the SMA Corporation of Cleveland, Ohio. The specific rotation of this sugar is 80.0 as compared with a theoretical value of 80.5 for pure galactose. The ratio of galactose to glucose required to reduce a given amount of Benedict's quantitative solution is 1.18: 1. No corrections have been made in our data for this difference in reducing power—all results are recorded as for glucose.

to maintain the animals on the expensive diets over long periods of time. Consequently, there were but few urinary sugar determinations in the galactose groups except where cataract development was delayed as in the Johns Hopkins and Wistar groups.

Rats remained on the lactose rations much longer and there was no difficulty in obtaining sufficient volume of urine for sugar analysis. Another difficulty was encountered in this group, however, because of accompanying diarrhea. Metabolism cages equipped with fine wire gauze a few inches beneath the coarse wire mesh floor of the cage held back all formed stools, and glass wool in the glass funnel retained smaller debris, but fluid stools could not be separated successfully from the urine. Where serious fecal contamination was evident the specimen was discarded, but where a small amount of sediment only was present the urine was clarified by centrifuging. The Myers ('24) micro-modification of the Benedict titration method was used for urinary sugar determinations and the fermentation procedure was carried out as described in a previous paragraph.

<center>DISCUSSION</center>

In attempting to evaluate and compare the blood or urine sugar figures obtained in this study, one is impressed with the wide variations and some inconsistencies. These are to be expected, however, under the conditions of the experiment, but the number of figures obtained in the several series here reported and the statistical treatment of the results give a strikingly significant and interesting picture.

Blood sugar. The observations summarized in table 1 show total blood sugar values well above normal in all groups on cataract producing rations. The apparent correlation of the susceptibility to cataract with the average blood sugar levels in the 35 and 25% galactose and the 70% lactose groups is striking, but its significance is lessened when one analyzes the figures for the individual animals in any one of these groups. The most rapid cataract development does not

TABLE 1

Blood sugar values and incidence of cataract

CARBOHYDRATE IN THE RATION	BREED	NUMBER OF RATS	TOTAL SUGAR		NONFERMENTABLE		FERMENTABLE		CATARACT[1]	
			Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Incidence	Development
				mg./100 cc.		mg./100 cc.		mg./100 cc.	%	days
35 G + 35 S	B.C.	15	34	274±6.1	28	172±6.7	28	102±4.0	100	16
35 G + 35 S	W.	14	31	264±3.4	17	185±5.3	17	79±4.9	75	35
35 G + 35 S	J.H.	14	29	275±5.6	14	195±5.9	14	86±4.7	90	30
25 G + 45 S	B.C.	44	70	246±8.0	48	141±3.7	48	113±2.8	94	22
25 G + 45 S + I-p (Lilly)	B.C.	7	13	252±8.4	8	170±5.5	8	103±4.9	100	22
70 L	B.C.	16	34	155±3.4	29	72±3.2	29	87±3.1	72	47
62 L	B.C.	15	29	164±4.9	15	73±3.1	15	92±5.1	29	66
35 X + 35 S	B.C.	2	8	119±5.6					0	..
35 F + 35 S	B.C.	2	9	103±5.8					0	..
70 S	B.C.	26	51	112±1.8					0	..

TABLE 2

Urinary sugar values and incidence of cataract

CARBOHYDRATE IN THE RATION	BREED	NUMBER OF RATS	TOTAL SUGAR		NONFERMENTABLE		FERMENTABLE		CATARACT[1]	
			Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Incidence	Development
				mg./24 hours		%			%	days
35 G + 35 S	W.	16	41	93±8.0	16	93±0.7			75	35
35 G + 35 S	J.H.	12	26	61±7.2					90	30
70 L	B.C.	37	95	63±5.3	59	86±1.0			76	39
70 L	M.S.C.	10	40	53±4.3	53	86±3.8			10	56
62 L	B.C.	12	23	35±2.9	12	82±1.5			29	66
70 S	All breeds }	35	56	0 (qual.)					0	..
		12	28	7±0.6					0	..

[1] Cataract data relate to the litters included in this study.

G = galactose, S = starch, L = lactose, X = xylose, F = fructose, I-p = Insulin-protamine (Lilly).

parallel the highest blood sugar levels nor do resistant individuals show a consistently lower blood sugar. It is conceivable that the duration of galactosemia may be quite as pertinent as its degree.

The question of the influence of breed upon galactose tolerance was a logical outcome of observations reported by Mitchell ('36) on breed difference in susceptibility to cataract. Average blood sugar values on three strains of rats fed the 35% galactose ration were strikingly similar, thus affording no explanation to account for the observed differences in cataract development. One Wistar litter which proved to be unusually resistant to cataract did show the lowest blood sugar of the rats of the same strain, but such litter variance was not consistent in other groups. In the Johns Hopkins strain the black-hooded rats developed cataract in one-third the time required for the buff-hooded, whereas the latter group showed galactosemia distinctly above the average for all rats of that strain. In none of these three strains were the blood sugar values as high or as variable as those reported by Day ('36) even though our observations included larger groups of animals. It is impossible to conclude, therefore, that the degree of galactosemia, as observed, is alone responsible for the developing lens opacities although there is undoubtedly a close relationship.

More interesting even than the level of the total blood sugar in the cataractous rats is the nature of this sugar. The difference between the total and the nonfermentable fraction (galactose) is assumed to be the blood glucose. Cori ('25) found that during the absorption of galactose there was no rise in the glucose content of the blood. Our present findings constitute a striking verification of this statement, although the conditions of the experiment were quite different. It is significant to compare the blood sugar of 112 mg./100 cc. in the starch control group with the fermentable fractions of the blood sugars in several of the lactose and galactose groups, which are all within the range of normal blood sugar figures. The fact that some of the latter are slightly less than normal

blood glucose figures may well be accounted for by the errors of experimental technic or may represent a true depression of the blood glucose level.

The nonfermentable fraction is in most cases responsible for the differences noted in total blood sugars in the respective ration groups. Thus it appears that the rapid absorption and slower utilization of galactose allows it to accumulate in the blood over and above the normal blood glucose which apparently is not greatly altered thereby. In fact it behaves like a soluble foreign body in the blood stream. The greater variability of the nonfermentable fraction is easily explained by differences in individual tolerance, by time of feeding, and by the total amount of food eaten. A nonfermentable sugar fraction was never found in the blood of starch-fed control rats.

A limited number of observations were made with other carbohydrate combinations; 35% fructose and 35% xylose, respectively, were substituted for a portion of the starch in the skeleton ration. When fructose was fed the blood sugar was normal and there were no eye changes. Xylose caused a slight rise in total blood sugar with a small nonfermentable fraction seeming to account for the increase. Ophthalmoscopic observations on the xylose group revealed some early and rather transitory lens changes which never progressed beyond this stage.

The lower incidence and slower development of cataract on lactose rations along with the usual diarrhea complication and retarded growth have resulted in curtailment of work with this sugar. The 62% lactose ration was employed with and without various additions (4, 6 and 8%) of calcium lactate. The original purpose was to observe whether excess calcium in a lactose ration would have any effect upon the development of lens opacities. These results were negative, but the calcium lactate tended to inhibit the diarrhea due to lactose and proved to be toxic at the higher levels. Blood sugar values under these conditions were entirely comparable to those obtained with the other high lactose ration and are of

interest here merely as further evidence of the partition of the sugar in the blood.

Consideration was given to the possibility of lowering the blood sugar to within normal limits while feeding galactose. Roe and Schwartzman ('32) and Mason and Turner ('35) have shown that insulin does not lower blood galactose in the human. Corley ('27) found that insulin did hasten the disposal of intravenously administered galactose in the rabbit. The possibility that the rat might respond differently could not be ignored. The problem of insulin shock and fluctuation of blood sugar level in a rat eating ad libitum eliminated regular insulin as a feasible agent. Some preliminary tests with the new Insulin-protamine (Lilly)[4] showed it to be slowly absorbed from the subcutaneous tissue of the rat. The action seemed to be prolonged for more than 24 hours and the maximum dose tolerated by young rats was 4 units administered every 48 hours. A limited number of observations were made on rats receiving a 25% galactose ration with graded doses of Insulin-protamine. No significant reduction in total blood sugar was observed and cataracts developed at approximately the same rate as in the control litter mates on the same ration without insulin. These findings would seem to confirm the observations of previous workers that insulin cannot lower blood galactose.

Urinary sugar. Both the type and amount of urinary sugar were determined on groups where this procedure was expedient. The comparative differences shown in table 2 for total sugar excreted in 24 hours are far less significant than for the blood sugar values because the size of the rat and the total food intake were not entirely comparable in the different groups. The severe diarrhea in young rats on lactose prevented the collection of satisfactory urine specimens at as early an age as was necessary in the galactose groups. Thus galactosuria was relatively much more severe on the galactose than on the lactose rations although the figures fail to emphasize this fact.

[4] Supplied through the courtesy of the Eli Lilly Company.

The type of sugar excreted was more significant than the total amount. The sugar remaining in the samples after 40 minutes yeast fermentation ranged from 82 to 93% of the total present before fermentation. Data are given in the table for four groups but other scattered determinations were invariable within the same range. The unavoidable dilution of the specimens in the course of the fermentation procedure and a possible decomposition of a trace of galactose are more plausible explanations of the 7 to 18% loss than the presence of an appreciable amount of fermentable sugar. Pure galactose solutions subjected to the same treatment showed similar losses in concentration.

Thus it may be concluded that galactose is the sugar responsible for both the high blood and high urine sugars observed in rats fed on lactose and galactose rations and must be the major etiological factor in this type of cataract.

SUMMARY

1. Blood and urine sugar studies have been made on rats fed on adequate rations containing 62 and 70% lactose, 25 and 35% galactose, 35% fructose, 35% xylose and 70% starch.

2. Determinations of total and nonfermentable sugar were made on both blood and urine specimens from the various ration groups.

3. Total blood sugar values were higher on galactose rations than on lactose but above normal in all animals on cataract producing rations.

4. The nonfermentable fraction of blood sugar was chiefly responsible for the differences in total blood sugar in the various groups, the fermentable fraction remaining more nearly constant and within the range of normal blood glucose.

5. Average total blood sugar values of three strains of rats fed on 35% galactose ration were strikingly similar, in contrast to the differences observed in susceptibility to cataract among the same groups.

6. A fructose-starch ration caused no hyperglycemia and no eye changes.

7. A xylose-starch ration brought about a slight elevation in blood sugar and some early transitory lens changes which did not progress beyond this stage.

8. Insulin-protamine (Lilly) failed to lower blood galactose or reduce the speed of cataract development on a 25% galactose ration.

9. A galactosuria of varying degrees was observed in all rats on lactose and galactose rations, relatively more severe in the latter and absent in starch-fed control groups.

10. It may be concluded that galactose is the sugar responsible for both the high blood and the high urine sugars observed in rats fed on lactose and galactose rations and must be the major etiological factor in this type of cataract.

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ANTITHYROGENIC ACTION OF CRYSTALLINE VITAMIN B [1]

BARNETT SURE AND KATHRYN S. BUCHANAN

Department of Agricultural Chemistry, University of Arkansas, Fayetteville

In a previous investigation Sure and Smith ('34) demonstrated that retardation of loss of weight can be secured in animals receiving toxic doses of pure crystalline thyroxin (Squibb's) by administration of a potent vitamin B concentrate.

The isolation of vitamin B(B_1) in pure crystalline form, supplied by Merck and Company, made possible a quantitative study of this problem. Various doses of thyroxin were given and an attempt was made to find the requirements of crystalline vitamin B to prevent loss of weight produced by hyperthyroidism. It was found that the efficiency of vitamin B in overcoming the toxicity of thyroxin depends largely on the source of the stable components of the vitamin B complex contained in the experimental ration. The results are summarized in tables 2 and 3.

Sixteen groups of rats in sets of four, litter mates of the same sex, were given graduated daily doses of thyroxin, 0.05 to 0.2 mg. with various daily doses of crystalline vitamin B, 1 γ to 160 γ daily. First, all animals were depleted on ration 1751, deficient only in the vitamin B complex. They were then transferred to ration 2345, deficient only in vitamin B, the antipellagric factor of which was furnished with 15% of autoclaved round steak, and dried at 100°C. This ration was supplemented with 4 drops of cod liver oil per animal per day.

In each experiment of this series the first animal served as the negative control, that is, it received a diet deficient only in vitamin B. The second animal received the same diet and thyroxin. The third animal received the B deficient diet, thyroxin and crystalline vitamin B. The fourth rat served as the positive control, which received vitamin B but no thyroxin. The gain in weight of the positive control was taken as 100%, and the gains in weight, if any, of the other animals in the group were then calculated accordingly. The

TABLE 1

Composition of rations

	NUMBER OF RATIONS		
	1751	2345	3080
Casein	20[1]	20[2]	20[2]
Agar-agar	2	...	...
Salts no. 185	4	4	4
Butterfat	10	10	10
Autoclaved beef[3]	...	15	...
Autoclaved Northwestern yeast[4]	...	...	15
Dextrin	64	51	51
	100	100	100

[1] Washed with acidulated water for 10 days.

[2] Washed for 10 days with acidulated water and extracted thoroughly with 25% alcohol.

[3] Autoclaved for 6 hours at 20 pounds pressure at the natural pH of the beef (round steak).

[4] Dehydrated baker's yeast supplied by the Northwestern Yeast Company, Chicago, Illinois. This yeast was autoclaved for 6 hours at 15 pounds pressure at the pH of 6.8.

incidence of protection was figured from the changes in body weight. For instance, if a positive control gained during the experimental period of 30 days, 24 gm., and if the litter mate that received thyroxin and vitamin B gained 12 gm. during this interval, that animal was considered as receiving 50% protection. Losses of weight were designated as zero protection.

It will be noted that on a daily dose of 0.05 mg. of thyroxin, 3 to 5 γ of B afforded 40 to 48% protection and 10 γ of B

TABLE 2

Antithyrogenic action of crystalline vitamin B on ration 2345

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE CRYSTALLINE VITAMIN B$_1$	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	PER CENT PROTECTION
		days	*mg.*	γ	*gm.*	*gm.*	*gm.*	
2991	♂-NC	17	....	...	74	56	—18	0
	L♂		0.05	...	88	64	—24	0
	R♂		0.05	1	89	63	—26	0
	D♂-PC		....	1	70	87	+17	100
2992	♀-NC	17	....	...	74	58	—16	0
	L♀		0.05	...	74	50	—24	0
	R♀		0.05	3	75	83	+8	48
	D♀-PC		....	3	62	79	+17	100
2993	♂-NC	14	....	...	63	39-D	—24	0
	L♂		0.05	...	65	51	—14	0
	R♂		0.05	5	55	65	+10	40
	D♂-PC		....	5	70	95	+25	100
2994	♀-NC	35	....	...	64	55	—11	0
	L♀	10	0.05	...	73	50	—23-DC	0
	R♀	35	0.05	10	62	113	+51	137
	D♀PC	35	....	10	44	81	+37	100
2984	L♂-NC	23	....	...	66	59	—7	0
	R♂	12	0.1	...	68	49	—19-DC	0
	D♂	23	0.1	1	59	55	—4	0
	DR♂-PC	23	....	1	60	82	+22	100
3004	D♀-NC	53	....	...	70	60	—10	0
	R♀	15	0.1	...	75	61	—14-DC	0
	DL♀	53	0.1	3	70	62	—8	0
	L♀-PC	53	....	3	67	95	+18	100
3006	L♀-NC	22	....	...	68	59	—9	0
	R♀	11	0.1	...	68	54	—14-DC	0
	D♀	40	0.1	8	65	78	+13	18
	DR♀-PC	40	....	8	63	133	+70	100
3007	♂-NC	37	....	...	80	66	—14	0
	L♂	18	0.1	...	80	62	—18-DC	0
	D♂	39	0.1	10	77	91	+14	18
	DL♂-PC	39	....	10	75	152	+77	100

TABLE 2—*Continued*

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE CRYSTALLINE VITAMIN B_1	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	PER CENT PROTECTION
		days	*mg.*	γ	*gm.*	*gm.*	*gm.*	
3008	♀-NC	21	....	...	90	64	— 26	0
	D ♀	29	0.1	...	90	67	— 23	0
	DL ♀	40	0.1	20	86	110	+ 24	42
	L ♀-PC	40	....	20	78	135	+ 57	100
3029	R ♀-NC	15	....	...	66	56	— 10	0
	D ♀		0.2	...	72	51	— 21	0
	L ♀		0.2	3	58	47	— 9	0
	♀-PC		....	3	83	83		100
3030	♂-NC	21	....	...	83	71	— 8	0
	L ♂	7	0.2	...	77	55	— 22-DC	0
	R ♂	13	0.2	5	65	49	— 16	0
	D ♂-PC	13	....	5	73	85	+ 12	100
3039	♀-NC	22	....	...	78	61	— 17	0
	L ♀	7	0.2	...	68	48	— 20-DC	0
	R ♀	15	0.2	8	70	52	— 18	0
	D ♀-PC	15	....	8	66	71	+ 5	100
3041	♂-NC	17	....	...	72	69	— 3	0
	L ♂	8	0.2	...	71	52	— 19-DC	0
	R ♂	11	0.2	10	65	54	— 11	0
	D ♂-PC	11	....	10	69	73	+ 4	100
3042	♀-NC	21	....	...	72	56	— 16	0
	L ♀	5	0.2	...	62	47	— 15-DC	0
	R ♀	10	0.2	20	67	56	— 11	0
	D ♀-PC		....	20	57	71	+ 14	100
3043	♀-NC	47	....	...	63	41	— 22	0
	L ♀	7	0.2	...	62	43	— 19-DC	0
	R ♀	11	0.2	80	74	74		0
	D ♀-PC	11	....	80	68	84	+ 16	100
3054	♀-NC	53	....	...	70	40	— 30-D	0
	L ♀	12	0.2	...	75	43	— 28-D	0
	R ♀	28	0.2	160	69	83	+ 14	32
	D ♀-PC	28	....	160	67	111	+ 44	100

NC = negative control; PC = positive control; D = died; DC = dying condition.

TABLE 3

Antithyrogenic action of crystalline vitamin B on ration 3080

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE N. W. YEAST	DAILY DOSE VITAMIN B$_1$	INITIAL WEIGHT	FINAL WEIGHT	CHANGE OF WEIGHT	PER CENT PROTECTION
		days	mg.	mg.	γ	gm.	gm.	gm.	
3140	R♂-C	42	...	500	...	100	214	+ 114	100
	L♂		0.2	500	...	100	159	+ 59	52
	♂		0.2	...	10	117	134	+ 17	15
3141	DR♀-C	42	...	500	...	111	180	+ 69	100
	DL♀		0.2	500	...	107	193	+ 86	124
	D♀		0.2	...	30	99	164	+ 65	94
3142	R♀-C	42	...	500	...	118	186	+ 68	100
	L♀		0.2	500	...	122	166	+ 44	65
	♀		0.2	...	50	110	160	+ 50	74
3143	DR♂C	42	...	500	...	122	241	+ 119	100
	DL♂		0.2	500	...	118	216	+ 98	82
	D♂		0.2	...	100	100	188	+ 88	74
3144	R♂-C	39	...	500	...	95	207	+ 112	100
	L♂		0.2	500	...	98	188	+ 90	80
	♂		0.2	...	10	92	154	+ 62	53
3146	R♀-C	39	...	500	...	99	165	+ 66	100
	L♀		0.2	500	...	101	186	+ 87	132
	♀		0.2	...	50	99	170	+ 71	108
3147	DR♂-C	39	...	500	...	76	221	+ 145	100
	DL♂		0.2	500	...	90	202	+ 112	77
	D♂		0.2	...	100	95	188	+ 93	64
3111	D♂-C	49	...	500	...	80	256	+ 176	100
	R♂		0.2	500	...	69	229	+ 160	94
	L♂		0.2	...	300	88	214	+ 126	71
	♂		0.2	...	100	90	230	+ 140	80

C = control.

produced a greater gain of body weight than the positive control. On a 0.1 mg. daily dose of thyroxin, however, as much as 20 γ of B allowed only 42% protection. The daily dose of 0.2 mg. of thyroxin is so toxic to animals on ration 2345 that even 80 γ daily of B produced no antithyrogenic action, and even on 160 γ of vitamin B, only 32% protection was secured.

Numerous experiments in this laboratory have demonstrated that considerably greater growth is obtained with graduated daily doses of crystalline vitamin B, using autoclaved Northwestern yeast, regardless of whether it was autoclaved at 15 or 20 pounds pressure, than with autoclaved beef, as a source of the stable components of the vitamin B complex. Since autoclaved beef to the extent of 15% in the ration supplies an abundance of the rat antipellagric factor, the dried baker's yeast must be furnishing a stable factor that is deficient in the autoclaved beef. Our observations, therefore, substantiate the claims of György ('35), Harris ('35), Chick and co-workers ('35), and of Elvehjem and associates ('36), that vitamin G (or B_2) is a complex consisting of at least two dietary essentials.

A series of experiments of eight groups were then investigated on a 0.2 mg. daily dose of thyroxin, with graduated daily amounts of crystalline vitamin B, 10 to 300 γ, on ration 3080, which contained 15% dried autoclaved baker's yeast (Northwestern) as a source of the vitamin G complex. In this study the animal of each group receiving a daily dose of 500 mg. of untreated Northwestern yeast, without thyroxin additions, was considered as the positive control. The graduated doses of crystalline vitamin B were then compared with 500 mg. daily of Northwestern yeast, in the presence of a daily allowance of a toxic dose of 0.2 mg. thyroxin. While there is considerable variation in the extent of antithyrogenic efficiency produced by the various doses of vitamin B, the fact that 64 to 94% protection has been secured with such small daily amounts as 30 to 100 γ of vitamin B, establishes the value of this vitamin as an antithyrogenic agent in experimental

hyperthyroidism. Furthermore, the greater efficiency of crystalline vitamin B on ration 3080 than on ration 2345, is due to the former ration furnishing a stable dietary factor deficient in the latter ration. We also found that the efficiency of rice polishings as an antithyrogenic agent is considerably enhanced on ration 2345 after it is supplemented with a daily allowance of 100 mg. of Lilly liver extracts (used in pernicious anemia) which is abundant in what was heretofore referred to as vitamin G, but which must be now regarded as a complex. Our interpretation is that the liver extracts supply a hitherto unrecognized stable factor of the vitamin G complex, probably synonymous with the new dietary factor of Elvehjem and associates ('35).

SUMMARY

After the stable components of the vitamin B complex have been provided for, which may be furnished by autoclaved Northwestern dehydrated baker's yeast, pure crystalline vitamin B_1 becomes an excellent antithyrogenic agent in daily doses of 30 to 100 γ to counteract the toxicity of as high a daily dose as 0.2 mg. of thyroxin.

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INFLUENCE OF HYPERTHYROIDISM ON VITAMIN A RESERVES OF THE ALBINO RAT [1]

BARNETT SURE AND KATHRYN S. BUCHANAN

Department of Agricultural Chemistry, University of Arkansas, Fayetteville

That various foodstuffs may exert a protective influence in experimental hyperthyroidism has been pointed out by Abelin ('30), Abelin, Knochel and Spichtin ('30). Von Euler and Klussmann ('32) observed that rats on a diet deficient in vitamin A and injected with thyroxin, suffered loss of weight less readily when also receiving liberal amounts of carotene. In 1935 Abelin reported that vitamin A can reduce the high basal metabolic rate produced in rats given toxic doses of thyroxin; also, that vitamin A administered as carotene will restore the reduced glycogen content of the liver in hyperthyroidism in the rat. Neither von Euler nor Abelin, however, was able to prevent the large losses of body weight caused by administration of large doses of thyroxin.

Since we found that vitamin B(B_1) acts as an efficient antithyrogenic agent only in the presence of an abundance of the stable components of the vitamin B complex (Sure and Buchanan, '37), we have introduced a diet composed largely of dried milk, as follows: Skimmed milk powder, 50; agar-agar, 2; salts no. 185, 4; ferric citrate, 0.2; butterfat, 10; and dextrin, 37.8. This ration was supplemented with 4 drops of cod liver oil per animal per day; in this study a daily dose of 0.2 mg. of thyroxin was employed. As a source of vitamin B we used Lilly vitamin B concentrate prepared for parenteral clinical use, kindly furnished by Mr. Rhodehamel of the Eli

Lilly Research Laboratories. This concentrate was supplied in ampules in liquid form, each cubic centimeter containing 150 Sherman-Chase vitamin B units. According to Waterman and Ammerman ('35) one Sherman-Chase unit is equivalent to 2.5 γ crystalline vitamin B. The units of vitamin B introduced by the Lilly concentrate were, therefore, translated in γ vitamin B.

The results are summarized in table 1. We were surprised to find that on this dietary regime, which furnished the same amounts of butterfat and cod liver oil as sources of vitamin A as ration 2345 (for composition see preceding paper), three animals, after showing excellent responses to Lilly vitamin B concentrate in antagonizing the toxicity of thyroxin, developed marked cases of xerophthalmia and died. One animal suffered with labored respiration, typical in the terminal stages of vitamin A deficiency.

It is true that a ration containing large amounts of dried milk powder to which fats have been added suffers vitamin A losses on standing (Sure, '29), but since this diet (3109) without thyroxin additions, furnished ample amounts of vitamin A for excellent growth, as is evident from table 1, the daily dose of 0.2 mg. thyroxin must have produced a depletion of the vitamin A reserves on the animals that succumbed with accompanying severe eye lesions after excellent responses have been obtained to vitamin B additions. In this respect we concur with the observations of Abelin ('35).

In six groups of rats an attempt was made to counteract the toxicity of a 0.2 mg. daily dose of thyroxin, when crystalline vitamin B proved ineffectual on ration 2345 (which supplied the stable components of the vitamin B complex in the form of autoclaved beef) with massive doses of carotene in oil, furnishing 500 units of vitamin A daily, given as 10 drops three times daily. Such massive doses of vitamin A produced no changes in body weight and showed no antithyrogenic value. Vitamin A even in massive doses cannot, therefore, replace the essential stable component furnished by autoclaved dried baker's yeast, and which is deficient in autoclaved beef.

TABLE 1

Antithyrogenic action of Lilly vitamin B concentrate on ration 3109

EXPERIMENT NO.	ANIMAL MARKINGS AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE LILLY B₁ CONCENTRATE	DAILY DOSE N.W. YEAST	INITIAL WEIGHT	FINAL WEIGHT	CHANGE OF WEIGHT	PER CENT OF PROTECTION	XEROPHTHALMIA[1]
		days	mg.	γ	mg.	gm.	gm.	gm.		day
3109	D ♂-PC	57	...	...	500	132	263	+131	100	27
	R ♂	57	0.2	...	500	123	180	+57	43	27
	♂	26	0.2	15	...	117	156	+39	30	27
	L ♂	57	0.2	30	...	117	98	−19-D	0	
		31	0.2		...	109	177	+68	52	27
		43	0.2		...	109	138	+29-D	22	
3112	D ♀-PC	35	...	...	500	112	160	+48	100	31
	R ♀	35	0.2	...	500	114	156	+42	88	
	♀	35	0.2	75	...	122	138	+16	33	
	L ♀	35	0.2	150	...	110	139	+29	60	
3113	D ♀-PC	56	...	...	500	122	195	+73	100	31
	R ♂	56	0.2	...	500	132	214	+82	112	
	♀	56	0.2	150	...	117	156	+39	53	
	L ♀	26	0.2	225	...	117	136	+19	23	
		34	0.2		...	117	97	−20-D	0	
3127	D ♂-PC	35	...	...	500	128	238	+110	100	27
	R ♂	35	0.2	...	500	137	212	+75	68	
	♂	35	0.2	225	...	138	208	+70	63	
	L ♂	35	0.2	300	...	140	202	+62	57	

[1] The xerophthalmia was accompanied by labored respiration.

PC = positive control; D = died.

SUMMARY

A diet containing 50% dried skimmed milk powder furnishes a sufficient amount of the stable components of the vitamin B complex, so that excellent responses are obtained to pure crystalline vitamin B as an antithyrogenic agent in experimental hyperthyroidism. Such a ration, however, containing 10% butterfat and supplemented with 4 drops of cod liver oil per animal per day, does not provide a sufficiency of vitamin A to counteract the rapid catabolism produced by a daily administration of 0.2 mg. of thyroxin.

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THE VITAMIN G [1] CONTENT OF SOME FOODS

HAROLD LEVINE [2] AND ROE E. REMINGTON

Department of Nutrition, Medical College of the State of South Carolina, Charleston

The experiments reported in this paper were carried out before it was established by the work of several investigators that a) what was hitherto called vitamin G is composed of at least two factors; namely, flavin and vitamin B_6, both these factors being necessary in the diet to permit normal growth and to maintain a normal condition of the skin in the rat (György, '35 a; Chick, Copping and Edgar, '35; Harris, 35; Copping, '36 a), b) neither flavin nor vitamin B_6 are effective in the prevention or treatment of human pellagra (György, '35 b; Spies and Chinn quoted by Birch, György and Harris, '35; Dann, '36), and c) assays employing the Bourquin-Sherman ('31) or the Munsell ('31) vitamin G-deficient diets measure the flavin content of a substance under test (Booher, Blodgett and Page, '34; Bisbey and Sherman, '35; Copping, '36 b; Day and Darby, '36). Some time before these facts were established, we determined the vitamin G content of various foods with a view toward locating inexpensive food sources which it was hoped would prove effective in combatting human pellagra in the south. In these tests, we employed both the Bourquin-Sherman and the Munsell vitamin G deficient diets which as stated above serve to measure the flavin content of a test material. Although we later learned that measurement of the flavin content is of no apparent value in determining the effectiveness of a food in the prevention or

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[1] In this paper, vitamin G refers to the factor flavin as determined by the Bourquin-Sherman vitamin G-deficient diet ('31).

[2] Now biochemist with the Premier-Pabst Corporation, Milwaukee, Wisconsin.

treatment of human pellagra, nevertheless these assays should be placed on record. Recent work indicates that flavin besides being a definite growth-promoting substance and concerned in the maintenance of a normal skin condition, also plays a role in food utilization and stimulation of the appetite (Griffith, '36).

The Bourquin-Sherman vitamin G deficient diet carries an 80% ethyl alcohol extract of whole wheat in such proportion that 100 gm. of the diet contain the extract of 50 gm. of whole wheat to serve as a flavin-deficient source of all growth factors of the vitamin B complex. The Munsell diet carries 30% white corn in place of the alcoholic wheat extract to serve the same purpose. Employing these two vitamin G deficient diets or modifications of them, several investigators (Booher, Blodgett and Page, '34; György, '35 a; Copping, '36 a, b; Day and Darby, '36) have demonstrated that flavin brings about renewal of growth when added to the diet of rats depleted on these rations. Elvehjem, Koehn and Oleson ('36) have recently reported their inability to produce growth in rats on a purified ration supplying vitamins B, B_2, B_4, B_6 and flavin unless another distinct factor which they have called the alcohol-ether precipitate factor is added to the diet. These facts taken together indicate that the above-mentioned vitamin G deficient diets contain all the factors needed to supplement flavin in exerting its growth-promoting effect.

That the Bourquin-Sherman diet, however, contains a limited amount of some factor or factors other than vitamin B and B_6 is evidenced by the findings of Booher, Blodgett and Page ('34) and Copping ('36 b). These workers employed multiples of the regular amount of alcoholic wheat extract used in the diet. Taking a low level of their flavin preparation as a supplement, Booher and co-workers ('34) found that "where growth is limited to 6.0 gm. per week, an increase in the level of wheat extract is of no advantage over our usual vitamin G deficient diet." However, when higher levels of flavin were fed yielding higher growth rates, the regular ration permitted only about 73 to 76% of the gain made by

animals on the reinforced diets. Copping ('36 b) worked only with a high rate of gain. When data for rats showing the same food intake are chosen from Copping's protocols, results similar to those of Booher and co-workers are secured. These findings can be interpreted to indicate that when flavin is the test material, the Bourquin-Sherman diet contains an adequate amount of some factor or factors (other than vitamin B_1 and B_6) for a rate of gain limited to 6.0 gm. weekly, but an insufficient amount when higher rates of gain are involved. Of course, when natural foods are assayed it is possible that data secured with higher growth rates may be reliable for calculation of vitamin potency, if it is known that the test food contains an abundance of the limiting factor or factors in the Bourquin-Sherman diet. In practically all the assays on natural foods reported in this paper, we have calculated the vitamin potency from data where the growth rate was limited to a gain of 3.0 to 6.0 gm. per week.

Several investigators (György, '35 a; Chick, Copping and Edgar, '35; Copping, '36 a and b) have shown that there are at least two types of skin lesions occurring in the rat, the 'specific' or 'acrodynia-like' type resulting from a deficiency of vitamin B_6, while the 'non-specific' type is brought about by a lack of flavin in the diet. Copping was consistently able to produce the 'non-specific' type of lesion in rats depleted on the Bourquin-Sherman diet and to bring about a cure of this skin derangement and restoration of growth by the administration of flavin.

From all the facts presented above it is clear, therefore, that the Bourquin-Sherman ration by virtue of a flavin deficiency permits the development of the 'non-specific' type of skin lesion and brings about cessation of growth in the rat, flavin curing the skin disorder and restoring growth. These facts are also presented here to show that assays employing the Bourquin-Sherman or Munsell vitamin G deficient diets serve to measure the flavin content of a test substance. At the time our assays were carried out, pure flavin was not available to us so that it was not possible to carry out parallel

feeding experiments with flavin in direct comparison with the foods which we tested. Our results, therefore, are based on the reliability of the feeding technic which we employed. Day and Darby have recently reported that 4 γ of flavin are equivalent to one Bourquin-Sherman vitamin G unit. However, until the growth-promoting property of pure flavin has been quantitatively established with the Bourquin-Sherman diet, it is desirable to express the flavin potency of foods in terms of Bourquin-Sherman units of flavin rather than in terms of weight of flavin. In our assays, therefore, we report the vitamin potency on this basis. Bisbey and Sherman likewise have used the Bourquin-Sherman diet to measure flavin values.

<div align="center">EXPERIMENTAL PART</div>

In looking about for sources of vitamin G, our attention was first drawn to cottonseed meal and soy beans, both inexpensive and abundant foodstuffs. Several investigators have reported that when cottonseed meal or flour (Osborne and Mendel, '17 a; Richardson and Green, '17; Hunt, '32) or soy beans (Daniels and Nichols, '17; Osborn and Mendel, '17 b; Salmon, '27; Wilkinson and Nelson, '31; Rittenger and Dembo, '32) constituted the only source of the 'vitamin B complex' in the diet, normal growth was permitted in the rat. These findings indicated that these foods are probably good sources of vitamin G and prompted us to assay these foods for their content of this vitamin. Since we were at the same time interested in knowing whether milk produced in a pellagrous area was as potent as that produced in other regions, we assayed a sample of dried whole milk produced in South Carolina. For comparative purposes, we also tested a sample of dried brewer's yeast used by the South Carolina State Board of Health in combatting pellagra. In addition to these tests, the solubility of vitamin G (as present in cottonseed meal) in 50% ethyl alcohol and the influence of pressure cooking on the vitamin G content of both cottonseed meal and soy beans was determined. In a preliminary experiment, the Munsell ('31) flavin-deficient diet was employed in assaying

the above-mentioned foods. Comparison of the results obtained was then made with the results of subsequent assays in which the Bourquin-Sherman ration was used.

Series I

The rats used in this preliminary series and in the following series of experiments were bred in our laboratory on a stock ration described elsewhere (Levine, Remington and Culp, '31). When the average body weight of the rats in each litter reached about 60 gm., they were then placed on the flavin-deficient ration described by Munsell. A total of forty-four rats were employed in the feeding tests. For about 2 weeks the animals gained in weight after which there was a tendency toward stationary or declining weight. After the body weight had become constant or had begun to decline for a period of 1 week (usually at the end of 21 to 25 days), various supplementary foods were given to the animals placed in individual cages equipped with raised wire screen bottoms. In some cases, mild alopeceia appeared at this time.

The supplements fed were cottonseed meal, soy beans (Biloxi variety), dried whole milk powder and dried brewer's yeast. The cottonseed meal and soy beans were purchased in the open market. The supplements were fed daily to each rat for a period of 8 weeks. The results obtained in our feeding tests are given in table 1. The negative control rats on the unsupplemented basal ration made an average gain of $+ 10$ gm. in body weight during the curative period. This finding agrees closely with that of Munsell and of Douglass and co-workers ('34) on the same ration. The Munsell diet is, therefore, not as free from flavin as the ration of Bourquin and Sherman which, according to growth curves given by these investigators and our own findings, permits an average loss of $— 4.0$ gm. during the 8 weeks test period. It is customary to express the vitamin G content of foods (Sherman, '32) in terms of Bourquin-Sherman 'units.' This 'unit' is based on the amount of food required to give a gain in weight of 3 gm. per week or a total gain in body weight of 24 gm. for an 8-week period,

when the Bourquin-Sherman diet is employed. When taken as the gain above the negative controls, 'unit' growth then becomes a gain of $24 + 4 = 28.0$ gm. Referring to the use of the Munsell ration for the determination of vitamin G and to calculation of the resulting data in terms of Bourquin-Sherman 'units,' Sherman ('32) states that "if under other conditions, as in the work described in 1931 by Munsell, the

TABLE 1

The vitamin G (flavin) content of some foods

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD		BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM [1]
			Actual gain	Gain above negative controls	
	gm.		*gm.*	*gm.*	*gm.*
Cottonseed meal	0.50	6	+ 51.0	+ 41.0	2.92
	1.00	6	+ 85.0	+ 75.0	
Soy beans [2]	0.50	6	+ 55.0	+ 45.0	3.20
Dried whole milk [3]	0.25	6	+ 47.0	+ 37.0	5.28
	0.50	6	+ 83.0	+ 73.0	
Dried brewer's yeast [4]	0.15	6	+ 94.0	+ 84.0	20.00
Negative controls on basal ration	0.00	7	+ 10.0	....	....

[1] One Bourquin-Sherman flavin 'unit' is equivalent to a gain of 28 gm. above the gain of the negative controls.
[2] Biloxi variety.
[3] Made from milk produced in South Carolina.
[4] Used by the South Carolina State Board of Health in combatting pellagra.

weight curve for the negative controls is appreciably different, the numerical 'values' should be corrected accordingly. For this purpose, a unit rate of gain in weight may be taken as 3.5 gm. per week above that of the corresponding negative control animals." On this basis, therefore, a gain of 28 gm. in 8 weeks above the gain of the negative controls represents one Bourquin-Sherman 'unit.' Applying this method of calculation to our data, we have expressed the flavin potency of the foods which we tested in terms of 'units' in table 1.

From a study of the results shown in table 1, it is evident that the foods investigated are all good sources of flavin, the order of decreasing vitamin potency in units per gram being: dried brewer's yeast, 20.0; dried whole milk, 5.28; soy beans, 3.20; and cottonseed meal, 2.92. In calculating the 'unit' values, we have assumed the gain in body weight (above the gain of the negative control rats) to be proportional to the dosage, our results with two levels each of cottonseed meal and of milk justifying this assumption. Where more than one level of the same food was fed, we have used the level yielding the gain nearest 'unit' growth for calculation of the flavin potency. The protocols of our assays also reveal that there was no significant diminution in the rate of growth when the gains in weight (above the negative control gains) were compared at the end of 4 and 8 weeks, indicating that throughout the test period the basal diet plus the supplement furnished an adequate supply of all growth factors for the rates of gain obtained.

In rats that had developed varying degrees of alopecia prior to feeding the supplements, cures of this condition were effected by the various foods tested. By the end of the test period the seven negative control rats, however, developed marked alopecia and other evidences of the 'non-specific' type of skin lesion described by Chick, Copping and Edgar ('35), György ('35 a) and Copping ('36 a, b). At the end of the curative period, the negative controls were continued on the basal ration for an additional 20 days during which time the skin and hair condition were even more characteristic of the 'non-specific' type of lesion. The basal ration was then augmented by the inclusion of 25% cottonseed meal. The animals were fed this ration for a period of 44 days, during which time the average body weight increased from 96 gm. to 206 gm.— a gain of 110 gm. The abnormal skin and hair condition of all the rats was cured and animals regained normal body vigor, demonstrating the ability of the flavin in the cottonseed meal to cure this type of skin disorder. During the period of cottonseed meal feeding, the average daily intake of the meal was 3.0 gm.

In some preliminary experiments designed to concentrate the flavin in cottonseed meal, we prepared a 50% (by weight) ethyl alcohol extract which we fed to one rat that had been kept on the basal ration for a period of 45 days. The extract was fed at a time when the rat was losing weight and exhibited a marked denuded skin condition. The animal was fed 0.3 gm. of the extract solids daily with the result that a gain in body weight of 30 gm. was effected in a period of 32 days, while the abnormal hair condition was completely cured. This preliminary finding demonstrated the presence of flavin in the alcoholic extract of cottonseed meal and prompted us to test the extract again on a larger number of rats.

Series II

The results described in series I led us to carry out further tests employing this time the Bourquin-Sherman flavin-deficient ration. We retested, therefore, the cottonseed meal and brewer's yeast with the Bourquin-Sherman diet in order to ascertain whether the two flavin-deficient rations would yield the same flavin values.

The alcoholic extract of cottonseed meal previously mentioned was also included in this series along with the residue from the extraction in order to determine what proportion of the vitamin was extracted and whether there was any loss in the process which was as follows: Five hundred grams of cottonseed meal[3] were extracted three times with 1000 cc. portions of 50% alcohol for 2 hours at room temperature. The extracts were separated by filtration under suction, combined, centrifuged free from suspended matter, and then evaporated to dryness on a steam bath. A brown sticky residue that became brittle and hard on cooling resulted. Eight such preparations using the same batch of cottonseed meal yielded an average of 66 gm. of extracted solids amounting to 14.2% of the cottonseed meal (dry basis). It was

[3] Upon analysis, the cottonseed meal was found to have the following percentage composition: Moisture, 7.43; fat, 7.45; crude fiber, 14.41; protein, 34.12; ash, 5.75 and N. F. E., 30.84.

determined that the third extraction removed only 8.5% of the extracted solids. The resultant cottonseed meal residue was dried at 60°C. in an electric oven.

The results of the assays are given in table 2. With the lower levels of the supplements, it was noted that not only was there a steady increase in body weight of the rats during the 8 weeks test period, but also that the total gains in weight were almost proportional to the level of the supplement fed.

TABLE 2

The vitamin G (flavin) potency of a) cottonseed meal, b) the 50% alcoholic extract of cottonseed meal, c) cottonseed meal residue and d) dried brewer's yeast

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD	BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM
	gm.		*gm.*	
	1.00	6	+ 63.0	
Cottonseed meal	0.50	8	+ 32.0	
	0.30	8	+ 21.2	2.94
50% alcoholic extract of cottonseed meal	0.40	7	+ 81.0	
	0.30	8	+ 65.0	
	0.20	8	+ 48.0	10.00
Cottonseed meal residue after extraction	1.00	5	+ 48.0	2.00
Dried brewer's yeast	0.20	6	+ 103.0	
	0.10	7	+ 51.5	21.4
Negative controls on basal ration	0.0	7	— 4.0	....

Bourquin and Sherman with whole milk powder and Poe and Gambill ('35 a and b) with tomato juice as supplements likewise secured similar findings where their supplements yielded comparable gains in weight. We also observed that wherever alopecia was present at the start of the curative period, it was cured in every case by the various supplements including the cottonseed meal extract. Such findings indicate that a combination of the basal ration plus the supplements contained an amount of all factors adequate for a rate of gain of at least 3.0 to 6.0 gm. weekly and sufficient to maintain a healthy skin condition in the rat.

As shown in table 2, a level of 0.30 gm. cottonseed meal gave a gain of + 21.2 gm. in body weight which was nearest to 'unit' growth of + 24.0 gm. By calculation at this level, a value of 2.94 Bourquin units per gram is obtained which agrees quite closely with the value obtained in series I. In the case of the solids of the alcoholic extract of cottonseed meal, we have also taken for calculation of potency the level yielding a gain nearest 'unit' growth. On this basis, the extract solids have a potency of 10.00 Bourquin units per gram, while the potency of the cottonseed meal residue was found to be 2.00 units per gram. The dried brewer's yeast was found to contain a potency of 21.4 units per gram which is comparable with the value of 20.0 units secured in series I

TABLE 3

The distribution of vitamin G (flavin) in the various cottonseed meal fractions

FRACTION	WEIGHT OF FRACTION	FLAVIN POTENCY (UNITS PER GRAM)	TOTAL UNITS IN EACH FRACTION
Cottonseed meal	*gm.* 500[1]	2.94	1470
Cottonseed meal alcoholic extract solids	66	10.00	660 ⎤
Cottonseed meal residue	397	2.00	794 ⎦ 1454

[1] Equivalent to 463 gm. moisture-free cottonseed meal.

with the Munsell ration. The extract solids are therefore 3.4 times as potent as the cottonseed meal, about one-half as potent as the yeast and about twice as potent as dried whole milk. From the results secured in series I and II, it can be concluded that both the Bourquin-Sherman and the Munsell flavin-deficient rations yield the same 'unit' values, when the difference in the body weight curves of the negative control rats on the two basal rations is taken into consideration in calculating the vitamin potency.

A vitamin G (flavin) balance sheet covering the various fractions resulting from the extraction of cottonseed meal was drawn up, yielding the results shown in table 3. From a study of this table, it is revealed that the entire potency of the cottonseed meal could be accounted for, about one-half of

the total flavin units appearing in the alcoholic extract, the remainder in the residue. No loss of flavin occurred during the extraction process.

Series III

In series I and II, the assays were carried out on uncooked cottonseed meal and soy beans. In preparing the soy bean for human consumption, it is customary to cook the bean, so that we felt it advisable to determine whether the cooking process brought about any destruction of flavin. Likewise, we felt

TABLE 4

The influence of pressure cooking on the vitamin G (flavin) potency of cottonseed meal and soy beans

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD	BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM
	gm.		*gm.*	
Uncooked cottonseed meal	0.6	8	+ 42.6	2.96
Pressure-cooked cottonseed meal	0.6	8	+ 32.0	2.22
	1.2	8	+ 65.0	
	1.8	8	+ 88.0	
Uncooked soy beans	0.4	8	+ 23.3	2.43
Pressure-cooked soy beans	0.4	8	+ 22.8	2.38
	0.8	8	+ 42.6	
	1.2	8	+ 65.0	
Negative control rats on basal ration	0.0	8	− 4.0	

that if cottonseed meal or flour were to find use as a food, such as an ingredient of bread, biscuits, etc., the effect of heating on the flavin content should be investigated.

Wilkinson and Nelson ('31) observed that when soy beans steamed for 3 hours are incorporated at a level of 10% in a diet devoid of vitamins B and G, normal growth results indicating at least partial stability to heat of the vitamins contained therein. In our experiments both soy beans and cottonseed meal were cooked in an autoclave for 30 minutes at a pressure of 15 pounds. The resulting preparations were assayed using the same feeding technic employed in series II. Both preparations were fed at the levels indicated in table 4.

In each case, the uncooked product was fed at one level to serve as a standard of comparison so that the extent of flavin destruction could be measured.

A study of the table reveals that at a level of 0.6 gm. of cottonseed meal, the uncooked product contained 2.96 units per gram, while the cooked preparation contained 2.22 units indicating that cottonseed meal when cooked as described suffered a loss in vitamin G content of only 25%. On the other hand, 0.4 gm. of soy beans, either cooked or uncooked yielded practically the same potency indicating that no loss of vitamin occurred in the cooking process. On the basis of these tests, therefore, flavin as found in cottonseed meal and in soy beans appears to be quite stable to pressure cooking.

The soy beans (Biloxi variety) used for the cooking tests were not from the same lot as that tested in series I and appear to be only about 76% as potent in flavin as the previous lot. The sample of cottonseed meal which was from the same lot used in series I and II gave practically the same 'unit' value as obtained in the two former series.

Here again, with the various levels of the cooked products, gains in weight practically proportional to the levels fed were secured, testifying to the presence of an adequate content of all growth factors in the combination of the basal diet plus the various supplements.

DISCUSSION

Although several investigators have determined the vitamin G content of milk and yeast, only a few have evaluated the potency of cottonseed meal and soy beans. Drawing his conclusions from indirect experimental data on rats, Stevens ('30) states that commercial cottonseed meal approximates yeast as a source of vitamin G. Essential details are lacking in the report published by this author. On the other hand, however, Sherwood and Halvorsen ('33) have found that cottonseed meal contains about one-eighth as much vitamin G as yeast. On the basis of our assays, cottonseed meal is about one-seventh as potent as the dried brewer's yeast which we

assayed simultaneously. Our findings are therefore quite at variance with those of Stevens and in good agreement with those of Sherwood and Halvorsen. Using the Bourquin-Sherman ration, Whitsitt ('33) fed a group of rats 2.4 gm. of cottonseed meal per week. By calculation using data taken from her growth curves, we find a potency of 3.2 units per gram which agrees closely with our value of 2.92 units. Munsell and Devaney ('33) report a value of 1.7 Bourquin units per gram for cottonseed flour, a finding which suggests that the meal is more potent than the flour and that in refining the meal some flavin is removed. Several investigators (Munsell and Devaney; Sherwood and Halvorsen; Whitsitt; Wilmot and Winters, '34) have reported that cottonseed meal or flour are good sources of vitamin B, one author (Sherwood and Halvorsen) reporting a value of 3 to 4 international units of vitamin B per gram for the meal. On the basis of our findings and the above reports, cottonseed meal can be considered a good source of both vitamin B and flavin.

With respect to the vitamin G potency of soy beans, Wan ('32) found that yellow soy beans contain two-thirds as much vitamin G as whole milk powder. In our assays (series I), soy beans were found to be approximately 60% as potent as dried whole milk, giving good agreement, therefore, with Wan's results. Wan also reports that soy beans contain three times as much vitamin B as dried whole milk. Drake-Law ('32) states that steam-processed soy beans are one-fifth as potent as dried yeast in vitamin G and one-tenth as potent as dried yeast in vitamin B. Since it is known that yeast varies considerably in vitamin B potency, it is impossible to determine from Drake-Law's report whether a high or low vitamin B potency for soy beans is indicated. Wan's finding, however, would indicate that the soy bean is a good source of vitamin B. Soy beans can, therefore, be considered a good source of both vitamin B and flavin.

The vitamin G values for cottonseed meal and soy beans obtained in our assays rank quite high when compared with the values given by Sherman for some common foods.

Sherman reviewing the findings of several workers gives as the vitamin potency of milk a value of 0.40 to 0.75 units per gram on a fresh basis. Recalculated to the dry basis, this value becomes 3.3 to 6.3 units per gram of dried milk. Stiebling and Alleman ('33) report a potency of 5.0 units per gram for skim milk powder; Morgan, Hunt and Squier ('35) a value of 5.6 units for dried whole milk, while Booher and Blodgett ('33) found 0.5 units per gram for fresh whole milk. Our value of 5.28 units per gram for dried whole milk, therefore, comes within the range of potency obtained by these investigators. It is apparent, therefore, that milk produced in a pellagrous area comes within the range of flavin potency of that produced in other regions. The results of our assay show that milk is richer in flavin than cottonseed meal or soy beans. It is about one-fourth as potent as dried brewer's yeast, a finding similar to that reported by Aykroyd and Roscoe ('29).

Munsell and DeVaney report a value of 17.0 vitamin G units per gram for dried brewer's yeast, while Morgan, Hunt and Squier obtained a value of 19.5 units, values which are to be compared with our finding of 20.0 to 21.4 units per gram for our yeast sample. The brewer's yeast used in combatting human pellagra in South Calorina is therefore a potent source of flavin.

Other workers have extracted dried baker's yeast and brewer's yeast employing concentrations of alcohol and other solvents quite close to the concentration that we employed in the extraction of cottonseed meal and have also obtained partial solubility of vitamin G in the solvent yielding results quite similar to ours. Sherman and Sandels ('31) found that 60% (by weight) ethyl alcohol extracted only one-half of the flavin content of dried baker's yeast, while Day ('34) who extracted baker's yeast with various concentrations of either acetone or methyl alcohol in water found that both 60% (by weight) acetone and methyl alcohol extracted only one-half of the flavin content of the yeast. Smith ('33) found about 50% of the potency in the residue when dried brewer's

yeast was extracted with 50% ethyl alcohol (by volume). This partial solubility of the flavin in cottonseed meal and in yeast may be due possibly either a) to concomitant partial insolubility of inorganic salts and protein in the solvent thereby permitting retention of some flavin by adsorption or b) to the existence of the vitamin in the food in both the bound and unbound or free form, only the latter form being soluble in the solvent. György, Kuhn and Wagner-Jauregg ('34) and von Euler and Adler ('34) found that the flavin in yeast is chiefly present in an undialyzable or bound form, combined with a protein carrier as a non-dialyzable 'flavo-protein,' while Supplee and co-workers ('36) suggest that flavin is not merely adsorbed on a substrate, but may be physically or chemically bound.

SUMMARY

1. The Bourquin-Sherman ('31) and Munsell ('31) vitamin G deficient diets were employed to measure the flavin content of various foods. Since pure flavin was unavailable at the time the assays were carried out it was not possible to make parallel feeding tests. The results are, therefore, based on the reliability of technic employed. Hence, the flavin potency of the foods tested is expressed in terms of Bourquin-Sherman units of flavin rather than in terms of weight of flavin.

2. Cottonseed meal, soy beans (Biloxi variety), dried whole milk and dried brewer's yeast were found to be good sources of vitamin G (flavin) and to contain 2.9, 2.4 to 3.2, 5.3 and 20.0 to 21.0 Bourquin-Sherman units of flavin per gram, respectively.

3. Milk from a pellagrous region (South Carolina) is apparently as potent in flavin as that from other localities.

4. Extraction of cottonseed meal with 50% ethyl alcohol (by weight) at room temperature removes only about half of the flavin content. No destruction of flavin occurred during the extraction process as evidenced by the fact that the remainder of the flavin was found in the residue. The solids of the alcoholic extract contained about 10.0 Bourquin-Sherman flavin units per gram representing a vitamin concentration of 3.4

times and a potency about one-half that of dried brewer's yeast and almost twice that of whole milk powder.

5. The flavin as found in soy beans and in cottonseed meal appeared to be quite stable to pressure cooking at 15 pounds for 30 minutes.

6. When differences in the body weight curves of negative control rats are taken into consideration in calculation of the flavin potency of a food, the Munsell and the Bourquin-Sherman flavin-deficient diets yielded almost the same 'unit' values for the foods studied.

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THE MINIMUM VITAMIN A AND CAROTENE REQUIREMENT OF CATTLE, SHEEP AND SWINE [1]

H. R. GUILBERT, R. F. MILLER AND E. H. HUGHES

Division of Animal Husbandry, College of Agriculture, University of California, Davis

A previous paper (Guilbert and Hart, '35) presented data on the carotene requirement of cattle. From these data, together with available information on other species, the generalization was made that vitamin A requirement is directly related to body weight rather than to energy requirement and that the minimum to prevent or cure deficiency symptoms and to permit normal growth of mammals was in the order of 20 to 30 micrograms daily per kilogram body weight. Before that paper was offered for publication, confirmatory evidence had been obtained with sheep and with swine; that is, doses of alfalfa meal, calculated on the basis of the generalization given above, the weight of the individuals, and the carotene analysis of the feed, uniformly cured the symptoms of deficiency. We now present data on the requirement of swine and sheep in terms of vitamin A as supplied by cod liver oil, in addition to carotene as furnished by alfalfa and solutions of the crystals in cottonseed oil. Experiments on the vitamin A requirement of cattle, using cod liver oil as the source, are also reported. These data are considered in relation to similar information on the rat.

METHODS OF PROCEDURE

Carotene analysis of feeds. The method of carotene analysis employed was a slight modification of the procedure proposed by Guilbert ('34). Briefly the procedure consists of subjecting the sample to saponification in aldehyde-free alcoholic KOH, and extraction with peroxide-free ethyl ether. The ether solution, after washing free from chlorophyllines, flavones and alkali, is distilled under reduced pressure, the residue taken up in petroleum ether and the xanthophyll removed by washing with 85 to 90% methanol. The resulting solution of carotene in petroleum ether, after making up to convenient volume is determined colorimetrically, using for comparison a dye solution prepared from orange G and naphthol yellow crystals. The dye solution was standardized against two samples of beta carotene having melting points of 182°C. and 184°C., respectively, that were prepared in the laboratory of the Carnegie Institution of Washington, Division of Plant Biology at Stanford University and supplied to us through the courtesy of Dr. H. H. Strain. Since no more carotene is extracted from the samples by other appropriate solvents nor by other procedures, the extraction is considered to be quantitative. Numerous determinations run with beta carotene, M.P. 182°C., and with the beta carotene added to alfalfa samples, have resulted in recovery of 95 to 98% of the added carotene when subjected to the routine procedure. Close agreement has been obtained on samples analyzed by various research and commercial laboratories that are using this method. That the biological response is proportional to the value found by carotene analysis was found through trials with alfalfa meals varying over tenfold in carotene content (Guilbert and Hart, '35).

Analysis of cod liver oil. A 30 gallon drum of medicinal grade cod liver oil, guaranteed to contain not less than 2100 U.S.P. 1934 revised units per gram, was obtained for these experiments. Analyses were run on dilutions of this oil and upon the unsaponifiable fraction with a Hilger Vitameter-A, equipped with a photographic attachment. The method of

saponification and preparation of the sample followed was that recommended in the report of the second conference on vitamin standardization (League of Nations, Quarterly Bulletin of the Health Organization, vol. 3, no. 3, September, 1934). The calculation of percentage vitamin A is based upon the value $E^{1\%}_{1\,cm.}$ 328 mμ = 1600 (Carr and Jewell, '33). Determinations on this oil at the beginning and at the end of the experiments showed no change in its extinction coefficient. The value found by this procedure was 0.083% vitamin A (Carr and Jewell concentrate). The 1934 conference on vitamin standardization recommended the factor 1600 for converting values obtained for $E^{1\%}_{1\,cm.}$ 328 mμ into a figure representing international units of vitamin A per gram of the material tested. On the basis of this calculation the cod liver oil contained 2120 units per gram, a value in agreement with the producer's guaranteed minimum.

Similar Vitameter-A analyses of U.S.P. reference cod liver oil which is assigned a value of 3000 units per gram, gave a value of 2070 biological units. This result is similar to the average value obtained with this instrument by eight collaborators and reported by Irish ('36). The discrepency in these results (aside from possible inherent limitations of the instrument) indicate that either the conversion factor or the U.S.P. reference cod liver oil is in error. Since this question is at present controversial (Bacharach, Drummond and Morton, '36) calibration of the Vitameter on the basis of the U.S.P. reference cod liver oil did not appear justified. We have, accordingly, used the value 0.083% vitamin A (Carr and Jewell concentrate) in the calculations of minimum vitamin A requirements reported in this paper, recognizing, however, that this value is subject to revision.

Very little difference was found in the Vitameter values obtained with dilutions of the oil used in these experiments or of the U.S.P. reference oil compared with their unsaponifiable fractions. Furthermore, the values found by the antimony trichloride reaction were roughly proportional to their respective Vitameter-A values. These results indicate that both

oils were comparatively free from interferring irrelevant material.

A quantity of oil from the batch used in these experiments has been saturated with CO_2 and stored at low temperature, thus providing a reference for reinterpreting the biological results, if within a reasonable time, a more satisfactory basis for evaluation becomes available.

Criteria used to determine minimum requirements. Investigations with cattle (Guilbert and Hart, '35) showed that night blindness was the first detectable symptom of deficiency to appear on a deficient ration and the last to disappear as the dosage of carotene was increased from sub-minimum to minimum levels. A level of carotene intake that just prevented night blindness sufficed also for excellent gains; the animals remained in thrifty condition for indefinite periods, though their storage was meager. Preliminary investigations with swine and with sheep demonstrated that the night blindness test applied equally well with these species, although occasionally, swine developed partial posterior paralysis before defective vision in semi-darkness had been demonstrated. The sheep, in our experiment, developed a partial optical impairment of a permanent nature in a shorter time after the appearance of night blindness than did cattle or swine. This eventually rendered half of our original number of sheep useless for the study of minimum requirement by means of the night blindness test. Numerous animals, particularly pigs, were autopsied soon after showing defective vision in semi-darkness. The unsaponifiable matter from 20 gm. of the liver tissue in these cases, concentrated to 2 cc. or less in chloroform solution, either failed to give any test with antimony trichloride or gave only a trace of blue color, thus showing that vitamin A reserves were practically exhausted. In the experiments with sheep and with cattle in which the number of animals was limited, the doses of vitamin A or of carotene were repeatedly increased and decreased until within reasonably narrow limits the least amount that prevented night blindness was found. Subsequently this level of dosage was held constant with

reference to body weight for periods varying from a few weeks to several months, and the amount of storage checked either by the time required for symptoms to reappear after the supplement was discontinued or by slaughtering the animals and determining the extent of storage in the liver by the antimony trichloride method. Night blindness consistently reappeared in such animals within 1 to 3 weeks after vitamin A therapy ceased; those slaughtered while receiving the minimum level had little storage as shown by the antimony trichloride reaction. While this procedure was also employed with swine, most of the data were obtained by placing different groups of animals on varying levels of vitamin A or carotene intake and maintaining these levels constant with reference to body weight throughout the experimental period. Examination of the liver extract by the antimony trichloride technic was made for all animals that died and for those slaughtered at the close of each experiment.

Practically normal gains, over long periods of time, have been made by animals receiving vitamin A or carotene in amounts that did not permit normal vision in semi-darkness. Apparently only when sufficient vitamin A is provided to meet the needs of all of the body tissues is there sufficient available to form adequate amounts of the visual pigments as elucidated by Wald ('34). Night blindness appears as the first evidence of want and the rapidity with which it may be cured is evidence that recovery is not complicated by repair of pathological tissue. The minimum intake to prevent it appears to represent a true physiological minimum, since the animals may make excellent gains and continue in a state of thrift for many months. The test applies equally well with young and with mature animals and to the different species under investigation. It is apparently conditioned upon a single physiological function of vitamin A, that is, the visual pigment cycle, as compared with the many complications of growth. The test in our experience is sufficiently sensitive so that the effect of varying the dosage of vitamin A over a range of 25% is consistently detected. It is relatively unaffected by such

factors as heredity, age, energy intake, and environmental factors that must be most rigidly controlled to assay within similar limits the vitamin A value of feeds by the growth method. The animals were tested by driving them about their lots in twilight, moonlight, or in dim electric light. Animals frequently show a remarkably memory of their surroundings and may run about quite freely even though sight is very defective. To avoid this error, barriers were placed in different locations to form a maze. By this means defective animals were readily detected and different degrees of night blindness noted. Swine also frequently demonstrated defective vision by being very difficult to drive, while normal animals moved about freely. The night blindness tests were made at least once and more often twice weekly and the observations recorded by four independent observers.

Rations used and method of feeding. The concentrate rations used are given in table 1, together with the total carotenoid pigment in the rations that was not extracted from petroleum ether solution by 90% methanol. Most of the pigment in the petroleum ether fractions of the swine rations, when passed through adsorption columns composed of equal parts of MgO and siliceous earth (method of Strain, '34), adsorbed strongly at the top of the column. A small portion separated and washed through quite readily, thus indicating that not all of the pigment was beta carotene. H. H. Strain (personal communication) has, as a result of his studies of the carotenoids of barley grain, confirmed our assumption that most of the petroleum ether fraction was beta carotene. Markley and Bailey ('35) have shown that the pigment remaining in the petroleum ether fraction from wheat grain is largely carotene. The colorimetric value that we found for barley grain is in good agreement with that calculated from biological tests with rats (Hughes, '33). The analyses indicate, therefore, the maximum contribution of vitamin A value from the basal rations and that no serious error results from considering the pigment as beta carotene for the purpose of these experiments.

For roughage in the cattle ration, wheat or barley straw was used. It had stood several weeks in the field after ripening before it was harvested, and contained no trace of green color. The extract from 100 gm. concentrated to small volume contained only a trace of yellow pigment. No yellow pigment was extracted by ether and alcohol from the cottonseed hulls that were used as roughage in the sheep rations. Since the

TABLE 1

Basal concentrate rations and their approximate carotene contents

COMPONENTS OF RATION	CATTLE	SHEEP	SWINE		
			No. 1	No. 2	No. 3
Dried molasses beet pulp	70.0	50.0			
Rolled barley	14.0	10.0	73.0	34.0	12.0
Cottonseed meal	15.0	30.0			
Brewers' rice				30.0	58.0
Wheat middlings			15.0	14.0	20.0
Wheat bran		10.0			
Tankage			10.0		8.0
Linseed meal				5.0	
Skim milk powder				15.0	
CaCO₃	1.0		1.0	1.0	1.0
NaCl	Ad lib.	Ad lib.	1.0	1.0	1.0
Carotene content milligram per cent[1]	< 0.02	< 0.02	0.02	0.02[2]	0.023

[1] These values represent the total pigment remaining in the petroleum ether fraction after extraction with 90% methanol and, as discussed in the text, is not all beta carotene.

[2] This ration, after substituting new for old crop barley, contained 0.067 mg.% 'carotene.'

concentrate rations were limited for cattle and sheep not to exceed 1.5 pounds daily per 100 pound body weight, the amount of carotene contributed by the basal rations was relatively insignificant and no correction for it has been made in the calculations. The swine rations, on the other hand, being higher in carotene and consumed in larger quantities per unit of body weight, contributed significantly toward the animals' requirements. The amounts furnished by the basal

rations daily per unit of body weight were, therefore, calculated and included in the total intake reported for the various
groups used in studying the minimum requirement of swine.

The cattle were fed individually in feeding stalls. The
dosage of cod liver oil was measured by a glass hypodermic
syringe, mixed with a small portion of the concentrate ration
and placed on top of the remainder of the concentrate allowance where it would be consumed first.

The sheep were fed in individual feeding stalls except
nine head that for a time were fed as a group. Alfalfa supplement was fed by mixing the meal with the concentrate
ration once daily. Both cod liver oil and solutions of carotene
in oil were administered by means of glass hypodermic
syringes graduated to 0.1 cc. that delivered 0.92 gm. of oil
per cubic centimeter at room temperature. For lower levels
of dosage of cod liver oil it was diluted with cottonseed oil.
The doses of oil and dilutions thereof varied from 0.5 to 2.5 cc.
daily. After the desired amount of oil had been drawn into
the syringe, the outside was wiped clean with a cloth. The
animal's head was drawn up, the mouth forced open and the
dose deposited on the back of the tongue, the syringe tip
being touched to the tongue to remove the adhering drop.
As the animals made no attempt to eject the oil, consumption
was considered to be quantitative.

Each group of pigs had access to a supply of basal ration
in self-feeders and feed records were kept on each group.
Alfalfa meal supplements were mixed with a small amount
of basal ration, the whole moistened to minimize loss, and fed
to the pigs individually in feeding stalls. Cod liver oil and
carotene solutions were administered in a manner similar
to that described for sheep.

The alfalfa meal used during most of the experiments was a
dehydrated product that had been in storage for nearly a year.
The carotene content of this dropped from 12.9 to 7.8 mg.%
carotene during the course of the experiments. The meal
used in the last experiment with swine varied from 17.6 to
18.7 mg.% carotene. The daily allowance for each individual

was weighed into paper bags at weekly intervals. The meal was sampled while the feeds were being weighed; and the composite sample, kept in a tightly covered container at — 5° to — 10°C., was analyzed for carotene at monthly intervals.

The crystalline carotene was obtained from the S. M. A. Corporation. The melting point was 165° to 168°C. Sufficient amounts for 1- and 2-week periods were dissolved in cottonseed oil and made up to volume so that doses varying from 1 to 2 cc. daily carried the desired amount of carotene. The solutions were analyzed colorimetrically for carotene, and doses calculated on this basis. Repeated colorimetric analyses of aliquots of 100 mg. samples of the carotene, dissolved in cottonseed oil and diluted with petroleum ether (usually 1 to 100), consistently gave a value of 90 to 94% of the quantity weighed, indicating an average of about 8% impurities. Adsorption and separation of the carotene from petroleum ether solutions on magnesium oxide, with subsequent elution and colorimetric determination of the fractions, showed that approximately 88% of the carotene was the beta form. To prevent deterioration, the cod liver oil and carotene solutions were kept in the dark at about 34°F., except at feeding time.

The sheep and swine were weighed at weekly intervals and the cattle were weighed fortnightly. The desired level of vitamin A supplement dosage was maintained practically constant with reference to body weight by adjusting it at each weighing period for any change in weight of the animals.

Cattle used and experimental procedure. Five mature cows that had been on vitamin A experiments for over 3 years were used in the experiments on minimum requirement of vitamin A supplied by cod liver oil. Their vitamin A intake had been controlled so that they could be depleted to the point of night blindness within a short period of time. One of these cows, a negative control, received no supplement of cod liver oil. In addition, a calf, 5 months old at the beginning of the experiment was used. This calf became night blind

while nursing its vitamin A deficient mother. As soon as the animals exhibited night blindness, varying doses of cod liver oil were administered until the approximate minimum level was found. This level of dosage was then kept constant relative to body weight for periods of 2 to 6 months. All of the cows were pregnant during the experimental period. Comments on storage and reproduction will be found in the discussion of results.

Sheep experiments. Depletion periods and procedure. Twenty-one head of 7-year-old ewes were used in a preliminary experiment to study their storage and rate of depletion of that storage on the basal ration. These animals previously had had favorable conditions for storage. Animals were slaughtered at the beginning and at intervals throughout the depletion period and the liver storage determined by the antimony trichloride technic. The initial storage varied from 2500 to 4000 blue units[2] per gram of liver. The liver storage of animals, slaughtered at bi-monthly intervals, declined as the depletion period progressed so that after 10 to 16 months of depletion, the liver values ranged from 200 to 600 blue units per gram of liver. Four remaining animals developed night blindness after 22 to 23 months on the basal ration. These died or were killed in cachexia after 27 to 30 months at which time no vitamin A could be demonstrated in their livers. The withdrawal of vitamin A from storage appeared more rapid during the first year than during the second, a finding analogous to that in cattle. The change in these animals from the onset of night blindness to death was very gradual and was chiefly characterized by anorexia, gradual loss of condition and muscular weakness. In the final stages, the corneas of two of the animals were partially clouded, but

[2] The antimony trichloride 'blue unit' is defined as the amount of vitamin A in 0.05 to 0.2 cc. of chloroform solution that will produce with 2 cc. of antimony trichloride reagent a faint blue color that approximately matches that produced by 1 microgram of carotene under similar conditions. The color also matches a copper sulfate solution equivalent to 0.5 mg. of copper per cubic centimeter. This is used as a standard for comparison. One blue unit on this basis approximates 0.25 international or U. S. P. unit.

sight in daylight was never seriously impaired. Pneumonia, enteritis and kidney involvement were the most conspicuous autopsy findings.

On July 25, 1934, thirty head of ewe lambs were placed in a bare lot and started on the basal concentrate ration and cottonseed hulls. One lamb, slaughtered at the beginning of the experiment, had 750 blue units of vitamin A per gram of liver, whereas two that were slaughtered after 3 and 7 months on the deficient ration, had liver concentrations of 1000 and 250 blue units of vitamin A per gram of liver, respectively. Similar lambs, slaughtered after corresponding periods on green pasture, had 1250 and 3300 blue units, respectively. The liver concentration of vitamin A, therefore, apparently trebled during 7 months on green pasture and reached a value approximating that of the aged ewes.

A ram was placed with the ewe lambs on October 26th and remained until December 1st. Eight of these females conceived. Night blindness first appeared in three of them on March 21, 1935, after 8 months on the deficient ration; and all twenty-seven of those remaining showed night blindness within the next month. Lambing coincided with this period. Of the eight lambs born, seven were weak and either died within a few hours without nursing, or, if they were able to nurse, died within 5 days, one lamb showing severe diarrhea. The liver extract of five of the lambs gave no color test with antimony trichloride, while two gave a trace of color. One lamb was strong and active at birth and was thrifty at 14 days when it was killed. Its liver contained 5 blue units of vitamin A per gram. The mother was one of the last to show symptoms of deficiency. Autopsy findings on the lambs were characterized by general enteritis and more or less congestion in the lungs. All the ewes appeared to have an abundance of milk.

The first eighteen ewes to develop symptoms were started on minimum requirement experiments, using the procedure outlined in the section on criteria used to determine minimum requirements. The last nine to become night blind were

turned onto green pasture, and all were normal at the end of a week, when their vision was tested in moonlight. Data on storage and reproductive history at the close of the recovery experiments are mentioned in the discussion of results. The time for depletion of storage in sheep was similar to that of cattle that had comparable vitamin A concentration in the liver as shown by the antimony trichloride test on animals slaughtered at the beginning of the depletion period.

Swine experiments. Over 100 head of Poland China and Duroc Jersey pigs farrowed in 1934 and 1935 were selected at weaning time, placed in paved lots and used in three sets of experiments involving basal rations 1, 2 and 3, respectively. In addition to these rations a small group of nearly mature hogs was fed for a short period on brewer's rice, a feed extremely low in carotene, supplemented by varying levels of cod liver oil.

Pigs, that received with their mothers, a normal ration including pasture until weaning at about 8 weeks of age required an average period of 146 days on basal ration no. 1 to become depleted. At this time the average weight was 167 pounds. Pigs whose mothers were restricted during lactation to a carotene low ration supplemented with 5% of fair quality chopped alfalfa hay, required from 70 to 120 days to become depleted on basal rations 2 and 3, respectively. Their average weight was about 85 pounds when symptoms of deficiency developed. Most of the pigs were started in the recovery experiments when they had shown night blindness on two or three successive nights. In some cases they were in more advanced stages involving diarrhea and varying degrees of muscular incoordination or partial paralysis. Although the response of these animals to vitamin A or carotene therapy was spectacular, recovery from partial posterior paralysis was usually incomplete.

Fifteen animals in prophylactic experiments involving different levels of carotene furnished by alfalfa meal confirmed the results of the recovery experiments as shown by the liver storage found at autopsy. The effect of a single 10-gm. dose

of halibut liver oil given by stomach tube was studied in four animals. One of these was autopsied after 48 hours. Vitamin A was demonstrated by the antimony trichloride test in the liver, kidney, lean muscle, abdominal fat and blood serum. The recovery was estimated at about 8% of the total ingested. The three remaining pigs grew normally and when they had attained a weight of about 200 pounds, 5 months later, still had a small reserve of vitamin A in their livers.

Fifteen control animals died or were killed in the early stages of deficiency. No vitamin A could be demonstrated in their liver tissues by the antimony trichloride test. During the course of the depletion periods a number of animals died and others were discarded because of unthriftiness. The storage of vitamin A in the livers showed that factors other than vitamin A deficiency were involved in these cases. Only animals that were thrifty during the depletion period were used. Data on fifty of these during the recovery experiments are summarized along with the data for cattle and sheep in tables 2 and 3.

RESULTS

To conserve space the records of individual animals and groups in the various experiments are all combined and presented in summary form in tables 2 and 3.

Table 2 presents the data on carotene requirement when the carotene was furnished by alfalfa meal and by crystalline carotene dissolved in cottonseed oil. The data for cattle from previous experiments are included for comparison. The results with the different species and with the two sources of carotene were practically the same. Twenty-five micrograms of carotene per kilogram body weight appears to be the minimum level that will meet the criteria employed. The data were obtained from mature animals that were maintaining their weight, from pregnant females and from rapidly developing young animals. No detectable difference in the requirement for these varied conditions was evident. Pregnant, non-lactating, cows maintained on minimum levels throughout

gestation, however, have uniformly given birth to weak calves that died soon thereafter. Increasing the carotene intake to three or four times the minimum during the last month of gestation has consistently resulted in normal calves at birth.

TABLE 2

Minimum carotene requirement of cattle, sheep and swine when supplied by alfalfa meal and by the crystals dissolved in cottonseed oil

SPECIES	NUMBER OF ANIMALS	SOURCE OF CAROTENE	CAROTENE INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT	RESULTS
Cattle[1]	10	Alfalfa	< 26	Remained night blind or partially defective
Sheep	14	Alfalfa	9 to 24	Two animals recovered on highest level, remainder continued night blind
Sheep	3	Carotene in oil	18 to 26	Remained night blind or partially defective
Swine	11	Alfalfa	16 to 22	Remained night blind or partially defective. Highest dosage borderline. Liver storage, negative to trace
Swine	2	Carotene in oil	19 to 20	Remained night blind, died, no storage in livers
Cattle[1]	10	Alfalfa	26 to 33	Recovered, small storage in liver, gains normal
Sheep	11	Alfalfa	25 to 35	Recovered, small storage in liver, gains normal
Sheep	3	Carotene in oil	27 to 36	Recovered, remained normal for 2 months. Symptoms recurred within a week after supplement was stopped
Swine	20	Alfalfa	25 to 39	Recovered, small storage in liver, gains normal
Swine	4	Carotene in oil	26 to 33	Recovered, remained normal for 3 months, small storage in liver

[1] Data from Guilbert and Hart ('35).

Similar treatment of sheep at the end of gestation in the present experiments did not result in any normal lambs. The results are complicated, however, by the fact that all of the ewes had manifested deficiency symptoms at various times

during pregnancy. No vitamin A could be demonstrated in the livers of any of the dead lambs.

Cows that had practically no reserve of vitamin A have been maintained for long periods during lactation on approximately the minimum level of carotene intake without developing any clinical symptoms of deficiency. The milk in these cases was practically devoid of vitamin A and nursing calves developed symptoms and died. Milk production was adequate to promote fair growth in the calves when they received a vitamin A supplement. In one case (a Shorthorn beef cow) in which milk and butter fat production records were kept, the milk production was not increased as the carotene intake was increased from the minimum level to prevent night blindness in the cow (15 mg. daily) to an intake of over 1 gm. daily, an amount in excess of that necessary to produce the maximum carotene content of butter fat for this animal. The butter fat production averaged about 0.5 pound daily.

Six sows that received slightly sub-minimum amounts of alfalfa supplement for several months, came into oestrum and mated more or less regularly, but no litters were produced.

Table 3 summarizes the results with cod liver oil. Although the results in terms of absolute quantities of vitamin A are subject to revision, they are, as with carotene, consistent for the different individuals and for the species under investigation. On the basis of the analysis employed, 6 to 8 micrograms daily per kilogram body weight were the least amounts that met the criteria for minimum requirements. In terms of the cod liver oil used, this represented doses of 0.36 to 0.48 cc. for each 100 pounds of body weight. Slight excesses appeared to result in more rapid accumulation of liver storage than was found under similar conditions with carotene.

Two non-lactating cows that were maintained on the minimum level of intake throughout most of gestation, were given between three and four times this amount during the last month. Both produced normal calves that remained thrifty and grew normally for 2 months while receiving their mother's milk. A third cow that had a prolapsed vagina 7 days before

normal calving date, was sacrificed and the calf delivered by Caesarian section. The calf was strong, active and appeared normal in every respect. Its liver contained 5 blue units of vitamin A per gram, a meager amount but not unusual in the newborn. Two cows were allowed to become deficient and developed symptoms during the last month of gestation. One aborted a 3-week premature fetus; the other had at term a weak calf that died the second day. The results with regard to reproduction are similar to those with carotene, except

TABLE 3

Minimum vitamin A requirement of cattle, sheep and swine using cod liver oil as the source and based on Hilger Vitameter-A analysis of the oil

SPECIES	NUMBER OF ANIMALS	VITAMIN A INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT	RESULTS
Cattle	2	4.3 to 5.6	Remained night blind, no improvement in 3 weeks
Sheep	5	3.1 to 4.1	Developed night blindness while receiving these amounts
Swine	3	3.7 to 4.4	Remained night blind for $2\frac{1}{2}$ months. Gains nearly normal
Cattle	5	6.7 to 8.4	Recovered. Remained normal for 2 to 6 months. Small storage in liver
Sheep	5	5.6 to 8.2	Recovered. Gains normal. Small storage on higher level
Swine	8	5.8 to 7.5	Recovered. Gains normal. Small storage in liver

like levels of carotene with respect to the minimum has resulted in deficiency in the calves within the first 3 weeks of the nursing period. This fact also indicates a somewhat more efficient utilization of preformed vitamin than of carotene.

Four ewes that received approximately three times the minimum level of cod liver oil during the last 2 to 3 weeks of gestation produced dead lambs or lambs that died within a few hours. No vitamin A could be demonstrated in the livers of the lambs by the antimony trichloride test. All of the ewes had shown symptoms of deficiency at various times during gestation, when the rate of dosage was being varied to determine minimum requirements.

All of the pigs were carried on the levels indicated in table 3 from the time of development of symptoms until they reached market weight (about 200 pounds). The average time was about 2½ months. The gains of those on the sub-minimum levels average 1.25 pounds daily; those on approximately minimum levels averaged 1.47 pounds daily, a very satisfactory gain for such animals. The liver storage of the latter varied from 3 to 12 blue units per gram, which represents a very small reserve.

TABLE 4

Summary of data on the minimum requirement of cattle, sheep and swine for vitamin A and carotene compared with the biological unit and other data for the rat

| SPECIES | SOURCE OF DATA | DAILY INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT | |
		Vitamin A	Carotene
Cattle	Author's data	6.7–8.4	26–33
Sheep	Author's data	5.6–8.2	25–35
Swine	Author's data	5.8–7.5	25–39
Rat	Bauman et al. ('34)	......	25
Rat	Biological unit[1]	4	6

[1] Based on the conversion factors recommended by the 1934 conference on vitamin standardization and the estimation that the rats average 100 gm. in weight during the test period.

DISCUSSION

In order to compare the information secured with cattle, sheep and swine with data on the rat and in the light of these figures to examine the hypothesis previously proposed that vitamin A requirement is directly related to body weight, table 4 is presented.

The 1934 conference on vitamin standardization defined the international unit as the vitamin A activity of 0.6 microgram of pure beta carotene. As a result of the comparison of thirty-five biological assays on twelve different materials with percentages of vitamin A determined spectrographically, 1 gm. of the Carr-Jewell concentrate, which is assumed to be nearly pure vitamin A, was assigned a value of 2.56×10^6 international units. One unit on this basis, therefore, equals 0.4 microgram of vitamin A (Carr-Jewell concentrate). These

values have been converted to the basis used for expressing the requirement of domestic animals by assuming that the rats used in biological assays average 100 gm. in weight during the test period.

It was further stated in the report on vitamin standardization "that daily doses of 2 to 4 international units of vitamin A when administered to young rats suitably prepared on a vitamin A-deficient diet, have been found adequate to restore growth." Twice the vitamin A biological unit dosage shown in table 4 coincides with the minimum found for domestic animals on the basis of the Hilgar Vitameter-A analysis of the oil. Four times the biological unit of carotene is comparable with the minimum that we found for carotene. Although this means of comparison is, for numerous reasons, open to criticism, the apparent agreement, nevertheless, appears to be significant. The data on carotene requirement is more direct and definite. Bauman, Riising and Steenbock ('34) found that 10 micrograms of carotene per week permitted moderate growth in rats while doses in excess of 20 micrograms per week did not enhance growth. Since their animals averaged about 115 gm. in weight during the experimental period, the average daily intake at the latter level was about 25 micrograms per kilogram body weight, a value that coincides with our data. The minimum requirement for carotene is also within the limits found for swine by Dunlop ('34). He states: "The vitamin A requirement of swine has been shown to be between 14 and 62 mg. of carotene per 100 pounds of ration." By calculation from the feed consumed and weights of animals in his experiments these limits represent an intake of 13 to 60 micrograms daily per kilogram body weight. The lower level supported nearly normal growth but deficiency symptoms persisted; on the higher level considerable storage occurred.

It has already been pointed out (Guilbert and Hart, '35) that the energy requirement per unit of weight of 100 gm. rats must be at least eight times that of adult cattle. Direct relation of vitamin A to energy requirement is, therefore,

contra-indicated. The agreement between species and between animals of the same species differing greatly in body weight leads to the conclusion that vitamin A requirement is directly proportional to body weight or to the weight of tissues that are highly correlated with body weight.

Vitamin A is known to be associated with nerve tissue and with epithelial structures throughout the body. It is involved in the visual process (Wald, '34). That vitamin A acts as an oxidizing catalyst has been used as a working hypothesis in some experiments (Euler, '31). It has been reported by numerous workers to be interrelated with thyroxine. It appears, therefore, that vitamin A is involved in the normal functioning of a variety of tissues and structures. The data on minimum requirement suggests that its function in the normal animal is related to the amount of these tissues rather than to their rate of metabolism.

Having been able to predict the carotene requirement of sheep and of swine on the basis of the generalization derived from the data on cattle and rats, and having found that the vitamin A requirement is also uniform with reference to body weight in all of these species, we are impressed with the possibility of extending the information to other mammals. Calculations from the clinical data of Hess, Lewis and Barenberg ('33) and from those cited by Garry and Stiven ('36) indicate that the human requirement for carotene and for vitamin A cannot be far different from that of the species studied. The calculations for the former were based upon the values for milk reported by Semb, Bauman and Steenbock ('34) and by Gillam ('34); for the calculations from later data, present conversion factors for biological units were used. A possible limitation of the prediction that the vitamin A requirement of mammalia is 6 to 8 micrograms per kilogram (on the basis of present criteria) and the carotene requirement 25 to 30 micrograms daily per kilogram body weight may exist in the carnivores that in nature are accustomed to receive this factor as preformed vitamin A. Some evidence in this direction is given by Frohring ('35) who found that considerably larger

doses per unit of weight of carotene were required to cure deficiency in puppies than in rats.

Although the requirements in terms of absolute quantities of vitamin A are subject to revision, it appears from the data presented that at low levels of intake, vitamin A and carotene approach biological equivalence, and that the ratio of their respective efficiencies widens as the dosage is increased. Davies and Moore ('34) and others have shown that much higher percentages of large doses of vitamin A are assimilated and stored than of carotene.

From the studies of storage at different levels of intake in animals it appears that five to ten times the amount necessary to satisfy our definition of the minimal intake, is a desirable minimum to set for practical purposes. Significant storage accumulates within a few months at such a rate of intake and a reasonable storage reserve is desirable. Even this factor of safety, however, results in the production of milk low in vitamin A value, at least when its source in the ration is carotene and when the reserves of the animal are low.

Our experience with different species of animals shows that, although the minimum requirement per unit of body weight is the same for young and old animals, the former are more susceptible to pathological manifestations during vitamin A privation. The young animal is particularly susceptible to severe diarrhea and while it almost invariably occurs in older animals the condition does not ordinarily become serious. When diarrhea is caused by the lack of this factor the recovery following vitamin A therapy is spectacular. Young animals are likewise more susceptible to nerve injury. Whether or not the apparently greater resistance of older animals to pathological changes in the tissues is due to inherent differences in the tissues or to the slow withdrawal of the last vestiges of reserves from adipose tissues must be left open.

SUMMARY

The results of experiments on the minimum vitamin A and carotene requirement of cattle, sheep and swine are reported. The night blindness test, supplemented by a check on storage either by depletion or by the antimony trichloride test on extracts of the liver tissue, was used as the criterion of sufficiency. Evidence was presented that the amount of vitamin A or carotene daily that just prevents night blindness, represents a physiological minimum. Carotene was furnished by alfalfa and by crystalline carotene dissolved in cottonseed oil. Vitamin A was supplied by cod liver oil. It was analyzed with a Hilger-Vitameter A and also by the antimony trichloride method and the results compared with similar analysis of U. S. P. reference cod liver oil. Limitations of expressing vitamin A in absolute terms are discussed.

The minimum carotene requirement for all of the species studied was found to be 25 to 30 micrograms daily per kilogram body weight, an amount in agreement with similar data on the rat. The minimum vitamin A requirement on the basis of the analysis and criteria used was found to be 6 to 8 micrograms daily per kilogram body weight. These figures also appear to agree well with data on the rat calculated from present conversion factors for biological units. Excellent growth occurred at these levels yet storage after extended periods was meager. The data support the hypothesis proposed by Guilbert and Hart ('35) that vitamin A requirement is directly related to body weight rather than to energy requirement and that the requirement of other species of mammals may be predicted on this basis. Calculations from clinical data indicate that the human requirement cannot be far different from that of the species studied. Indications of poor use of carotene by puppies is cited as a possible limitation in applying the generalization for carotene to carnivores.

Data on depletion periods for swine and sheep together with observations on reproduction are presented and discussed.

ACKNOWLEDGMENT

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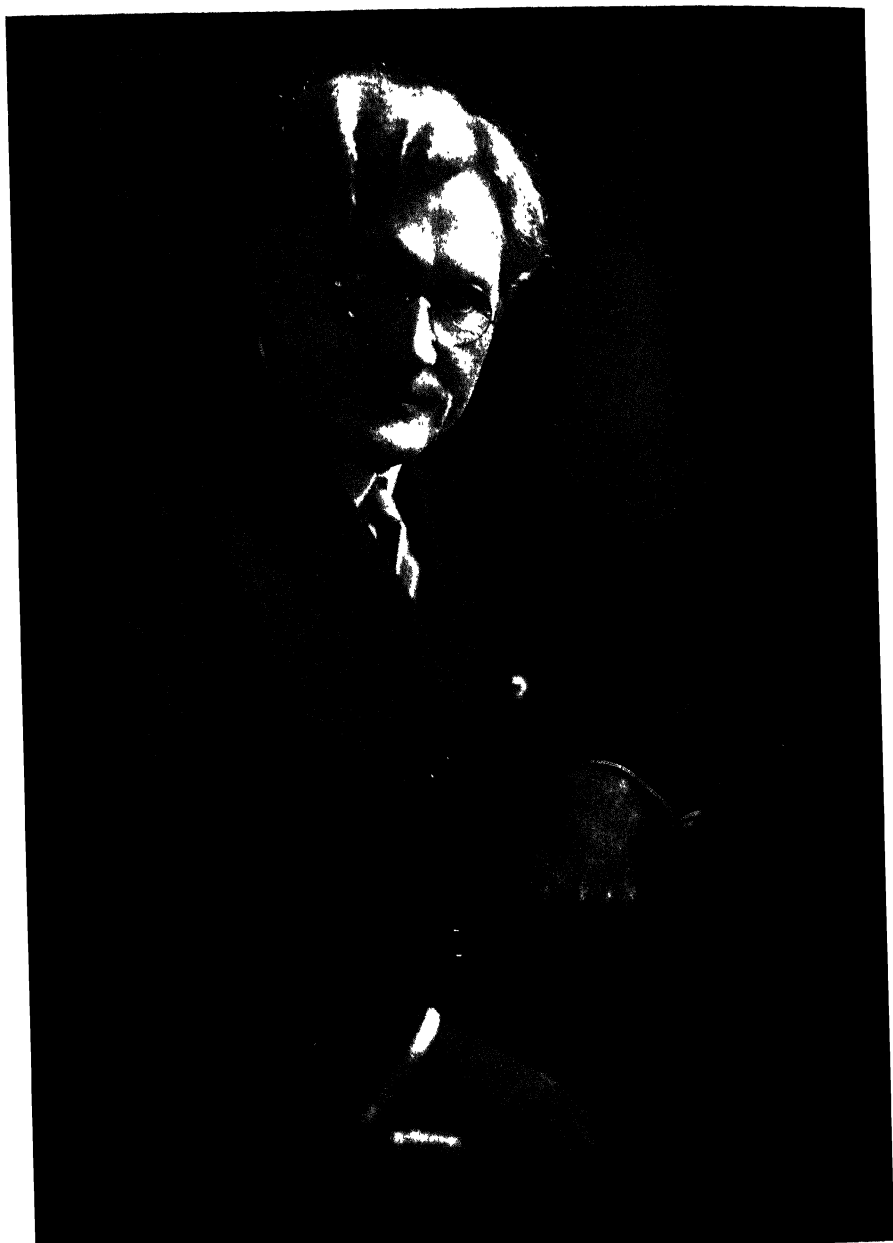
W. F. Greenman

EDITORIAL

Milton Jay Greenman

On April 7, 1937, passed away this rare personality and capable administrator of The Wistar Institute of Anatomy and Biology. Doctor Greenman gave evidence early in his career of being a man of unusually liberal ideas. An institute which in the beginning was a museum has evolved under his guidance into a large establishment for breeding of standardized animals, a valuable publishing enterprise, and a research institution with a biological farm as an adjunct, which is the first of its kind in the world. Only a mind receptive to new ideas and an order of administrative ability quite out of the ordinary could have blended and integrated these various interests of the Institute into a successfully working unit. It is not too much to say that The Wistar Institute can now provide standardized research animals of many kinds for biological investigation, can supply laboratory equipment and housing in which to carry on the investigations and finally publish the results to the world in any one of eight journals 'all under one roof' so to speak. This singular combination of resources is unique in research institutions.

Readers of The Journal of Nutrition probably are not as familiar as they should be either with the present character of The Wistar Institute or with the man who has been chiefly responsible for its metamorphosis. Doubtless more adequate memorial publications regarding Doctor Greenman will appear in due time. The following data have been gathered from material supplied by Miss G. L. Lawton of the Institute Staff.

Doctor Greenman was born at North East, Erie County, Pennsylvania, on June 14, 1866. He graduated from the college of the University of Pennsylvania with the Ph.B.

degree in 1889, and from the School of Medicine with the M.D. degree in 1892. University of Pittsburgh conferred on him its honorary Sc.D. in 1912 and the University of Pennsylvania the same degree in 1927.

He was assistant instructor in general biology, University of Pennsylvania, 1887 to 1889; instructor in biology, 1889 to 1892 while pursuing his medical studies; and lecturer on physiology, Biological School, University of Pennsylvania from 1892 to 1893.

He became Assistant Director of The Wistar Institute in 1892 under Dr. Harrison Allen, the first director. Upon Doctor Allen's resignation in July, 1894, Doctor Greenman continued as Assistant Director with Dr. Horace Jayne until the resignation of Doctor Jayne in December, 1904. Doctor Greenman was elected Director of The Wistar Institute in January, 1905.

The respect in which Doctor Greenman was held as an experienced administrator is indicated by the demand upon his services as trustee of the following institutions: Marine Biological Laboratory, Woods Hole, Massachusetts; Laboratory of the Biological Survey of the Mount Desert region, Bar Harbor, Maine; Vineland Training School, Vineland, New Jersey; Yale Laboratories of Primate Biology, Orange Park, Florida; also as a member of the Council and Publication Committee of the Philadelphia Academy of Natural Sciences.

Membership in the following societies indicate the breadth of his interest as well as his competency in science: American Society of Naturalists, American Association of Anatomists, Eugenics Research Association, Academy of Natural Sciences of Philadelphia, American Association for the Advancement of Science (Fellow), the College of Physicians of Philadelphia (Fellow). Honorary societies which conferred membership on him were Sigma Xi, and the American Philosophical Society. He was a corresponding member of the Peking Society of Natural History, an Honorary member of the Sociedad Cubana de Historia Natural, 'Felipe Poey,' 'First Patron' of the Anatomical and Anthropological Association of China.

Doctor Greenman was a competent investigator, although his preoccupation with administrative duties prevented full fruition of his talents in the research field. Articles on the regeneration of peripheral nerves appeared from his pen in The Journal of Comparative Neurology ('13, '17) and in the Journal of Nervous and Mental Diseases ('16). Several technological articles appeared in The Anatomical Record ('07, '08, '15). The remainder of his publications concerned his beloved Wistar Institute (Bulletin nos. 1, 3, 5, 6, 7, 8 and articles in the Proceedings of the American Association of Museums 1912, 1913; the Rockefeller Series on methods and problems of Medical Research 1930; the University of Pennsylvania General Magazine and Historical Chronicle, 1931).

Doctor Greenman was always first to disclaim credit for all the advances made by The Wistar Institute under his directorship. A few days after his succession in 1905 he called a conference of representative anatomists of the country for the purpose of outlining ''a policy whereby the Institute might participate to a greater degree in the promotion of American biological research.'' This resulted in the establishment of an Advisory Board as a permanent feature of the Institute's organization. The original ten were the following, G. Carl Huber, Lewellys F. Barker, Henry H. Donaldson, Edwin G. Conklin, Franklin P. Mall, J. Playfair McMurrich, George S. Huntington, Charles S. Minot, Simon H. Gage and George A. Piersol. This Board (naturally changing as to personnel) has met regularly once a year, usually at the Institute, to discuss its scientific policy and make recommendations to the Board of Managers. In Doctor Greenman's own words the Advisory Board has suggested continuously ''the type and field of research which the Institute could most effectively follow.'' [1] He credits the Board, and in particular Prof. E. G. Conklin, with the idea of having The Wistar Institute give support to scientific publications. Through the Board's ''efforts the several publications issued by the Institute have

[1] Bulletin no. 6 of The Wistar Institute, Commemorating the Twentieth Anniversary of the Organization of the Advisory Board, 1925, p. 36.

been brought together under one management," and the Institute has been brought into closer cooperation with various societies supporting the journals. Doctor Greenman commends "the peculiar wisdom of extending the field of research of an existing institution rather than establishing a new one" [2] adopted by the Board of Managers; also the wisdom of establishing its own press equipment, whereby a deficit in 1922 of nearly $12,000 in the cost of publishing its six journals, was converted into a profit in 1924 of $582. To readers of this Journal the last comment will be of particular interest because it explains how a research institution can print and publish under its own roof superior journals at less cost to its subscribers than can a commercial press. Economies are effected not only by adopting a uniform format for several journals and by preparing copy in a uniform manner, but also in the purchase of stock and in the amount of clerical work required per journal.

While Doctor Greenman very generously credits these policies to the two Boards with which he was constantly associated, the members of the Boards in turn at every opportunity gave full credit to Doctor Greenman. For example, Mr. Effingham B. Morris, recent President of the Board of Managers in his introductory remarks at the twentieth anniversary celebration referred to above, said, "I would like to say a word (before introducing him) about Doctor Greenman. You know he is the most modest man that you can find in a day's walk. In addition to all this work that you know about . . . . there are many other things that Doctor Greenman has been directing in his own quiet way." [3] Then he went on to speak of a line of investigation in which one would not expect the Wistar to be engaged.

Likewise Dr. H. H. Donaldson in his report on the research activities of the Wistar at the 1925 celebration said, "if we have succeeded, in a measure, in carrying out our research program, it has been largely accomplished through the constant cooperation of the Director of the Institute—Doctor

[2] Ibid p. 38.
[3] Supplement to Bulletin no. 6, p. 9.

Greenman—a natural investigator temporarily engaged in administering a scientific institute." [4]

Dr. C. E. McClung in speaking of the "Changes in Method of Biological Research as pursued by Museums and Possibilities of the Future," said "It is possible to adopt the autocratic method and put in charge a man of strong personality and intense convictions and authorize him to carry forward a personal program. This gets results of a definite kind and is an efficient method, but it depends upon one man and is limited by his ability and productivity. There is the other method of reaching out and making numerous contacts and thereby profiting from the abilities of many people, while at the same time a corresponding return is made to them. This is not so easy as the one-man method, because the involvement of numbers means the increase of mutual adjustments and that necessarily complicates matters. But it can be done, and The Wistar Institute has shown the way by its last twenty years of operation. As a result it has had an influence all out of proportion to its size, because, through the foresight of its Director, it elected to render the service that many desired, rather than the one which he determined." [5]

Readers of the Journal may not be aware that Doctor Greenman had been for many years intensely interested in the subject of nutrition, and it was this interest that made him immediately receptive to the idea first expressed to the writer by Dr. Lafayette B. Mendel, member of our Editorial Board, that The Wistar Institute purchase the Journal. The details of this transaction have been related in volume 7, page 365 of this journal. It was also through his efforts that a Department of Nutrition has recently been established in a small way in The Wistar Institute. All of our readers will be interested in the further progress of this department.

The chief pride of the Director in recent years has been the development of The Effingham B. Morris Biological Farm near Bristol, Pa. A visit will well repay students of nutrition, for there is already in operation a rat colony building, an

[4] Bulletin no. 6, p. 51.
[5] Ibid p. 72.

opossum colony and colonies of various amphibian forms—
all of which would lend themselves admirably to investigation
of many problems in nutrition. The idea of this farm origi-
nated from the necessity of supplying standard feeds for the
rat colony in Philadelphia. It has however grown far beyond
the original conception, due quite obviously to the open-
minded enthusiasm of Doctor Greenman for new and progres-
sive ideas.

How The Wistar Institute Managers will meet the
emergency created by Doctor Greenman's death remains to
be seen. It is the personal opinion of the writer that any
change in personality of the Directorship will not materially
alter the pleasant associations already existing between the
Editorial Board and The Wistar Institute. The publication
policy has been so successful that it is sure to be continued
substantially along the lines which now exist.

Doctor Greenman, however, will be sorely missed. His
charming manner and infectious enthusiasm for the various
projects of the Institute were of great influence in securing
harmonious cooperation. One of his admirers writes, ''No
one can take his place whether in ability or in sympathy and
help extended to young workers. I owe much to him and his
encouragement. The only way is to pass it on to other young
workers.''

<div align="right">JOHN R. MURLIN</div>

HEMOGLOBIN REGENERATION IN ANEMIC RATS IN RELATION TO IRON INTAKE

WITH SUGGESTIONS FOR IMPROVEMENT OF THE BIOASSAY TECHNIC FOR MEASURING AVAILABLE IRON

MARGARET CAMMACK SMITH AND LOUISE OTIS

Department of Nutrition, University of Arizona, Tucson

ONE FIGURE

Interest in the determination of the availability for hemoglobin formation of the iron in foodstuffs has resulted from the finding that all compounds of iron are not equally effective in the remission or prevention of nutritional anemia (Elvehjem et al., '33). The bioassay method of measuring the extent to which the iron in a food is utilizable for hemoglobin consists in comparing the gains in hemoglobin in anemic rats fed a food source of iron, with the hemoglobin regeneration in litter mate anemic rats given the same amount of iron as iron chloride, both food and iron chloride being amply supplemented with copper. In the method as developed by Elvehjem et al. ('33), the comparison is made at a 0.3 mg. daily level of iron feeding, an amount of available iron which has been found to raise the hemoglobin to the normal level in a 6-week test period.

In the course of our bioassay measurements of the hematopoietic value of food stuffs, we encountered the difficulty of measuring the availability of iron in foods of low iron content due to the inability of the rat to consume a large enough portion to obtain the required 0.3 mg. of iron daily.

As pointed out in a previous paper (Smith and Otis, '36) it does not seem necessary to compare the availability of iron in a food with Fe as $FeCl_3$ at this particular level. It is logical to believe that as long as the iron content of the food portion fed is not higher than is necessary to promote complete regeneration in the test period, it should be possible to make comparisons of availability at any iron level which is present in a readily consumable portion of the food to be tested—that is—at iron levels below that which is necessary for complete regeneration of hemoglobin. Accordingly, it seemed advisable to measure the hemoglobin regeneration in young anemic rats at different levels of iron intake, and the results of this study are reported in this paper.

EXPERIMENTAL PROCEDURE

Preparation of the test animals. Following the procedure developed by Elvehjem and Kemmerer ('31), young rats which had access only to whole milk, were weaned at 3 weeks of age and continued upon the milk ration until a definite degree of anemia developed. The degree of anemia was determined by hemoglobin measurements (using the Newcomer method) of blood obtained from the tail. Young rats in our laboratory handled according to the Elvehjem-Kemmerer technic, except that the litters were not reduced in size, reached an average hemoglobin level of 3.9 gm. per 100 cc., usually between the fourth and fifth week after weaning. The supplemental iron or food feeding was then begun and the regeneration in hemoglobin measured bi-weekly during the subsequent 6-week test period. In every case 0.05 mg. of copper as $CuSO_4$ and 0.04 mg. of manganese as $MnCl_2$ were given in addition to the iron or the food supplement. The iron was fed as a solution of iron chloride whose iron content had been determined by the thiocyanate method of analysis (Kennedy, '27). It was measured directly from a micro-burette onto a small portion of powdered milk which the rats consumed greedily. The iron free manganese chloride and copper sulfate supplements were given at the same time. The animals were housed

individually in heavily galvanized metal mesh cages with raised screen bottoms. Whole milk and distilled water provided in glass containers were given ad libitum.

The gains in hemoglobin at levels of iron intake varying from 0.014 mg. to 0.3 mg. daily, appear in table 1, and are shown graphically in figure 1.

TABLE 1

Hemoglobin gains in anemic rats fed different amounts of iron as iron chloride

AMOUNT OF IRON FED DAILY [1]	NUMBER OF RATS USED IN STUDY	INITIAL HEMOGLOBIN	GAINS IN HEMOGLOBIN (GRAMS PER 100 CC.)		
			In 2 weeks	In 4 weeks	In 6 weeks
			± Probable error	± Probable error	± Probable error
A. Males					
mg.		*gm. per 100 cc.*			
0.014	14	4.0	0.4 ± 0.16	0.7 ± 0.07	1.1 ± 0.18
0.028	13	3.9	1.0 ± 0.08	1.6 ± 0.12	2.2 ± 0.25
0.050	20	4.0	1.3 ± 0.11	2.4 ± 0.13	3.5 ± 0.14
0.071	5	3.9	1.7 ± 0.30	3.3 ± 0.27	4.3 ± 0.24
0.10	14	4.0	2.4 ± 0.16	3.8 ± 0.13	5.5 ± 0.14
0.15	11	4.0	3.5 ± 0.17	5.6 ± 0.11	8.2 ± 0.21
0.20	12	3.8	3.8 ± 0.12	6.7 ± 0.21	9.1 ± 0.19
0.25	12	3.5	5.5 ± 0.17	8.4 ± 0.15	10.4 ± 0.18
0.30	7	4.1	5.1 ± 0.30	8.6 ± 0.27	10.1 ± 0.29
B. Females					
0.014	5	4.1	1.0 ± 0.20	1.8 ± 0.30	2.3 ± 0.32
0.028	14	4.1	1.5 ± 0.13	2.5 ± 0.12	3.1 ± 0.24
0.050	13	4.4	2.1 ± 0.25	3.0 ± 0.29	4.2 ± 0.33
0.071	15	3.9	2.8 ± 0.17	3.9 ± 0.11	5.6 ± 0.25
0.10	12	4.0	2.7 ± 0.09	5.0 ± 0.17	6.5 ± 0.15
0.15	10	3.75	4.4 ± 0.22	6.6 ± 0.27	9.5 ± 0.26
0.20	8	3.7	5.0 ± 0.27	8.4 ± 0.22	10.9 ± 0.25
0.25	6	4.1	5.7 ± 0.30	9.0 ± 0.28	10.7 ± 0.18
0.30	7	4.6	6.3 ± 0.30	9.3 ± 0.17	10.2 ± 0.16

[1] 0.05 mg. Cu as $CuSO_4$ and 0.04 mg. Mn as $MnCl_2$ given daily in addition to the iron supplement.

Inspection of table 1 reveals the following points: As reported previously from this laboratory, a difference between males and females in their response to the supplementing of their whole milk ration with iron may be noted, hemoglobin regeneration being greater in the females than in the

males given the same amount of iron. This difference between the sexes was consistently noted at the end of the 6-week test period except at levels of iron feeding greater than 0.2 mg. daily. That complete hemoglobin regeneration up to the
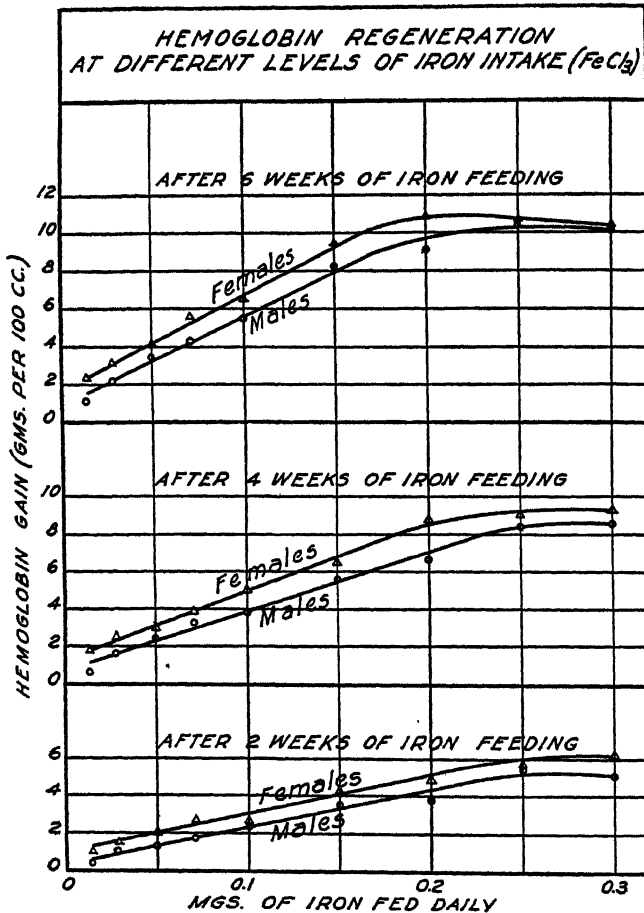
Figure 1

level normal for rats of this age resulted in males from the feeding of 0.25 mg. of iron daily and in females from the feeding of 0.2 mg. daily iron was evident by the finding that no greater gain in hemoglobin resulted from a higher intake

of iron. With daily iron intakes of 0.25 mg. or more, therefore, no observable differences between the sexes in their response could be expected. At the end of 4 weeks, however, sex differences in hemoglobin gains were observed even at the higher levels of feeding for the regeneration had not reached the maximum level in the shorter period of time.

It may be noted that the magnitude of difference in the hemoglobin gains of males and females was about the same at all levels of iron feedings. It is also apparent that it was in the first 2-week period after the beginning of the iron supplementation that the females showed the greatest hemoglobin gains as compared with the males. In the last 2 weeks of the 6-week test period, the differences were very slight. These facts suggest that the difference may be due to a greater store of iron in the female which became available upon the feeding of the copper given with the iron supplement. It may be noted, however, that the initial hemoglobin concentration, i.e., that at the beginning of the test period, is 4.0 for the ninety females studied and 3.9 for the 108 males. Thus a difference in reserve supply of iron between the males and females was not indicated by a significant difference in the hemoglobin concentration of the blood.

With both male and female anemic animals, at levels of iron below that necessary to induce complete regeneration of hemoglobin, the regeneration of hemoglobin was found to be directly proportional to their supplemental iron intake, although not arithmetically so, for the gains in hemoglobin are greater in proportion to the amount of iron fed at the lower levels of iron intake. It required a greater amount of iron to produce the same increment of hemoglobin gain at the higher levels of iron feeding. For example, when the iron level was raised from 0.014 mg. daily to 0.028 mg. daily, an increase of 0.014 mg. of iron, the gain in hemoglobin in the males increased from 1 to 2.2 gm. per 100 cc. of blood. On the other hand, increasing intake from 0.15 to 0.2, an iron increase of 0.05 mg. resulted in no greater increase (from 8.2 to 9.1) in hemoglobin than that resulting from 0.014 mg.

of iron at the lower level of intake. The relationship between iron intake and hemoglobin gain appears to be practically a linear one between the levels of iron feeding of 0.028 to 0.15 mg. per day. It would appear, therefore, that the anemic rats are more sensitive, i.e., make greater hemoglobin gains in proportion to the iron intake, at iron levels less than 0.15 mg. daily, for at iron levels above 0.15 mg. daily the hemoglobin gains in proportion to the amount of iron fed declined.

Obviously, the extent of regeneration of hemoglobin also varied with the length of the feeding period. At no level of iron feeding tested had the hemoglobin regeneration reached its maximum value in less than 6 weeks. At the end of both 2 and 4 weeks of supplementary feeding, the gains in hemoglobin, though smaller than the 6-week gains in general, are directly proportional to the amount of iron given. The results were, however, less regular after only 2 weeks of iron feeding and the increment of hemoglobin gain per unit weight of iron fed was appreciably less. However, the 4-week gains are quite uniform, and the increment of hemoglobin gains per unit weight of iron were not very much smaller than those obtained at 6 weeks.

DISCUSSION OF RESULTS

In view of tabulated results, the authors of this paper make the following suggestions for the improvement of the Elvehjem method for the measurement of the available iron in foodstuffs by bioassay.

That comparisons of the gains in hemoglobin in anemic rats fed a food source of iron with the hemoglobin gains in litter mate anemic rats given the same amount of iron as iron chloride be made at iron levels below 0.2 mg. of iron daily, preferably in the range of 0.03 to 0.15 mg. daily feeding. The exact level selected should depend upon the amount of iron present in a readily consumable portion of the food under test.

It is obvious not only that more uniform results are obtained when the amount of iron fed is below that necessary to promote complete normal regeneration of hemoglobin, but that

smaller differences in iron availability may be detected as the hemoglobin response per unit weight of iron fed is greater when less than 0.3 mg. of iron is fed daily. At levels of iron feeding of 0.2 mg. or above, the use of a shorter test period in which maximum hemoglobin development is not possible would be preferable. At all levels of iron, a 4-week test period gives results which are quite as uniform as those obtained in a 6-week test period. However, as the increment of gains in hemoglobin per unit weight of iron is somewhat smaller at 4 weeks than at 6 weeks, there is a slight sacrifice of sensitivity of the measurement in selection of the 4-week period. The saving of time resulting from a shortened test period is always an argument in its favor.

Again, as females show a greater gain in hemoglobin than males fed the same amount of iron, it is important that males and females should not be used interchangeably, but that comparisons of iron availability should be confined to members of the same sex.

The authors also suggest the possibility of the use of the curves presented herein, which show graphically the hemoglobin gains resulting from the daily feeding of iron in a completely available form at different levels to male and female anemic animals prepared for the test as herein described, as reference curves from which the amount of available iron in a given food supplement can be read from the curve if the hemoglobin gain resulting from the daily feeding of that food is known.

Table 2 presents the results of measuring the availability of the iron in certain foodstuffs using the Elvehjem method modified as suggested above. Measurements of the availability of iron in both corn and peas have been made at two different iron levels (table 2). It may be seen that when the resulting gains in hemoglobin in each case are compared with the hemoglobin gains obtained by animals of the same sex when the iron in the same amount as that present in the food supplement (see reference curve, fig. 1) was fed as $FeCl_3$ the observed percentages of availability of the iron are substantially the same at both levels of iron feeding. It appears,

TABLE 2

Availability of iron in certain foods

FOOD TESTED	GRAMS OF FOOD SUPPLEMENT	MILLIGRAMS OF Fe[1] IN FOOD SUPPLEMENT	6 WEEKS GAIN IN HEMOGLOBIN (GRAMS PER 100 CC.)		MILLIGRAMS OF Fe AS $FeCl_3$ REQUIRED FOR OBSERVED GAIN IN HEMOGLOBIN		PER CENT AVAILABILITY BY BIOASSAY		PER CENT AVAILABILITY BY DIPYRIDYL
			♂	♀	♂	♀	♂	♀	
Dried peas	2.0	0.1154	5.5	6.7	0.10	0.098	87	85	81
Dried peas	1.0	0.0577	...	4.3	....	0.051	...	88	81
Ground whole corn	4.0	0.0960	4.9	...	0.088	....	92	96	..
Ground whole corn	2.0	0.0480	...	4.0	....	0.046	...	86	..
Dried lima beans	1.5	0.1003	4.8	6.1	0.084	0.086	84	86	93
Ground whole wheat	3.0	0.1569	7.7	9.1	0.148	0.145	94	92	92
Ground rolled oats	3.0	0.1056	5.7	6.8	0.105	0.100	99	95	96
Ground whole barley	3.0	0.1230	4.3	5.2	0.073	0.069	59	56	..
Banana powder	4.0	0.0980	5.6	6.6	0.102	0.096	104	98	95–100[2]
Raw potato	4.0	0.0284	...	3.1	....	0.028	...	98	95–100[2]
Ground rye	3.0	0.1090	6.0	7.0	0.111	0.104	101	95	..
Dried apricots	2.0	0.2220	6.9	...	0.130	....	58	...	98[2]
Raw cabbage	4.0	0.0134	...	2.0	....	0.008	...	60	82[2]
Raisins	4.0	0.1378	...	6.5	....	0.094	...	68	94–97[2]
Dates	4.0	0.0204	1.0	...	0.012	....	59	..	82

[1] Iron analyses made on several samples of each food by thiocyanate method.

[2] As determined by Shackelton and McCance. Biochem. J., vol. 30, p. 582 ('36).

therefore, that this method may be used in determining the availability of the iron in foods of low iron content.

Although the percentages of available iron in the food under test as determined by this method agree closely in most foods with the availability as determined by the dipyridyl method introduced by Hill ('30) and modified by Shackelton and McCance ('36), the available iron as determined by bioassay was found to be notably less in certain foods than that found by the chemical method for determination of ionizible iron. These foods mentioned in this table include apricots, raisins, cabbage and dates. The relative low availability of iron in these foods as actually found by feeding experiments may be due to their relatively high roughage content which serves as a mechanical obstruction to the absorption of the iron. Whatever the cause of the lower availability obtained by bioassay tests, which measure the actual utilization of the iron in the food by the animal body, such measurements would logically be the stronger test of physiological availability.

It may again be noted that the hemoglobin gains resulting from the feeding of the different foods is greater in the females than in the males fed the same food and in the same amounts. However, if comparisons are made between those receiving the food and those rats of the same sex receiving the same amount of iron as iron chloride, the percentage availability of the iron in the food may be seen to be practically the same in males and females. However, let it be noted that if care is not taken to make the comparisons of availability of food iron and iron as iron chloride in animals of the same sex the results would be very different. For example, if the hemoglobin gains of the females fed 3 gm. of wheat daily were compared with the response of the males fed the same amount of iron as iron chloride, 115% of the iron in the wheat would appear to be available. On the other hand, if the response of the males fed the food source of iron was compared with the expected hemoglobin gains of the females given iron in its completely available form, a percentage availability of only 72 would be recorded. Thus it is quite important that this sex difference be recognized in making these comparisons.

If this difference in response between males and females can be explained by assuming a larger store of iron (i.e., Josephs' ('32) mobile tissue iron) in the female which cannot be used for hemoglobin formation because of exhaustion of copper reserves, and which therefore becomes available when copper or a food containing it is fed, then at least part of the hemoglobin regeneration resulting from the feeding of a food would be due to its copper, not iron, content. The necessity of exhaustion of body reserves of iron has been previously stressed by Elvehjem and his co-workers. It seems to the authors, therefore, that a more accurate procedure would be to deplete the iron reserves in the presence of sufficient copper so that the hemoglobin response in the test anemic animals would be a measure only of the utilization of iron. The practicability of this procedure and its effect upon the results is being investigated further.

SUMMARY

Hemoglobin regeneration in anemic rats in relation to their intake of iron given as iron chloride has been measured and the results used as the basis for modification of the method for determining available iron in foods by bioassay.

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THE EFFECT OF GALACTOSE ON THE HUMAN RESPIRATORY QUOTIENT AND ALVEOLAR CARBON DIOXIDE [1]

THORNE M. CARPENTER

Nutrition Laboratory, Carnegie Institution of Washington, Boston

The effect of galactose on the human respiratory quotient has been well established by Deuel ('27), Cathcart and Marko-witz ('27), and Carpenter and Lee ('32 a, '32 b and '32 c). In general, there is a marked rise in the respiratory quotient (R.Q.) within one-half to three-quarters of an hour with a sharp fall soon after, and later a gradual return to the pre-ingestion level. As the R.Q. may rise from an average basal level to above unity, the explanation that the rise is due to the increase of combustion of the carbohydrates cannot suffice. Several investigators have found increases in the lactic acid formation after the ingestion of galactose. Wierzuchowski and Laniewski ('31) found an increase in the lactic acid content of the blood and an increased excretion of lactic acid into the urine during continuous intravenous injection into dogs, Ørskov ('32) noted an increase in blood lactic acid after ingestion of the sugar by dogs, and Koike ('34) observed a marked increase in blood lactic acid after intraperitoneal injection of galactose into rabbits.

In view of the possibility of an increase of lactic acid after the ingestion of galactose, a determination of the changes in alveolar CO_2 in parallel with the changes in R.Q. seemed to

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offer a possibility of finding an explanation of the rise in the
R.Q., in part, at least. Several series of experiments have
been made with a long trained human subject in which the
percentage alveolar CO_2 and the respiratory exchange with
particular reference to the R.Q. have been determined before
and after the ingestion of galactose, galactose and glucose,
and lactose. A series was made with 250 cc. of water as a
control, a series each with 25 and 50 gm. of galactose, one
with 25 gm. of galactose and 25 gm. of glucose combined, and
one with 50 gm. of lactose. The sugars were given in 250 cc.
of water. The order of periods was three 15-minute periods
before ingestion of the dose followed by twelve 15-minute
periods consecutively after the dose was taken. The alveolar
air samples were taken every 7 to 8 minutes during the
periods. The respiratory exchange was measured by the open
circuit method of Carpenter and Fox ('31). The alveolar air
was collected by a method previously described (Carpenter
and Lee, '33 a). The subject was J.C., 52 years of age, 68.5
kg. in weight, and 167 cm. in height. He had served as a
subject of a similar investigation of the effect of glucose and
fructose on the alveolar CO_2 and the R.Q. (Carpenter and
Lee, '33 b). As in the previous investigation, the experiments
were made with the subject in a sitting position and therefore
they were not made in a strictly basal condition.

RESPIRATORY QUOTIENT AND ALVEOLAR CARBON DIOXIDE

The results of the determinations of the R.Q. and of the
alveolar CO_2 for the five series are plotted in figures 1 to 5.
The R.Q.'s are plotted at the middle points of the periods and
the alveolar CO_2 percentages at the points of time at which
they were taken. The quotients and the percentages for each
experiment are plotted with the same convention. The solid
lines in each figure represent the averages of all the observa-
tions of the R.Q. for the respective periods and the averages
of the percentages of the alveolar carbon dioxide. Table 1
gives the correlation coefficients between the R.Q.'s and the
accompanying average alveolar CO_2 in the respective periods

TABLE 1

Correlation coefficients between respiratory quotient and alveolar carbon dioxide of J.C.

DATE	NUMBER OF PERIODS	RESPIRATORY QUOTIENTS			ALVEOLAR CARBON DIOXIDE			CORRE-LATION COEFFI-CIENT	P.E.$_r$
		Average	Range	S.D.	Average, per cent	Range, per cent	S.D.		
250 cc. water									
Dec. 12, 1934	15	0.788	0.759–0.806	0.011	6.31	6.23–6.49	0.077	−0.182	±0.168
Dec. 14, 1934	15	0.789	0.757–0.802	0.011	6.05	5.92–6.26	0.089	+0.052	±0.174
Dec. 31, 1934	15	0.810	0.788–0.833	0.013	6.04	5.88–6.20	0.083	−0.304	±0.158
Jan. 9, 1935	15	0.800	0.779–0.838	0.016	5.99	5.81–6.18	0.082	+0.264	±0.162
Jan. 18, 1935	15	0.809	0.793–0.836	0.013	6.14	6.00–6.28	0.068	−0.256	±0.163
Average	15	0.799	0.775–0.823	0.013	6.11	5.97–6.28	0.080	−0.085	±0.165
25 gm. galactose									
Dec. 10, 1934	15	0.809	0.771–0.897	0.038	6.07	5.80–6.18	0.102	−0.465	±0.137
Dec. 28, 1934	15	0.822	0.775–0.936	0.051	5.91	5.77–6.12	0.119	−0.706	±0.087
Jan. 4, 1935	15	0.847	0.794–0.975	0.058	5.83	5.59–6.06	0.146	−0.471	±0.136
Jan. 11, 1935	15	0.869	0.815–1.007	0.059	6.03	5.71–6.22	0.145	−0.730	±0.081
Jan. 14, 1935	15	0.893	0.837–1.014	0.057	6.17	5.97–6.32	0.111	−0.828	±0.055
Average	15	0.848	0.798–0.966	0.053	6.00	5.77–6.18	0.125	−0.640	±0.099
50 gm. galactose									
Jan. 16, 1935	15	0.883	0.804–0.982	0.067	5.91	5.54–6.14	0.150	−0.607	±0.110
Jan. 28, 1935	15	0.873	0.798–0.975	0.066	5.86	5.62–6.13	0.153	−0.605	±0.110
Jan. 30, 1935	15	0.888	0.790–0.996	0.070	5.76	5.41–5.90	0.141	−0.782	±0.068
Feb. 4, 1935	15	0.899	0.818–1.000	0.069	5.98	5.75–6.19	0.132	−0.625	±0.106
Feb. 6, 1935	15	0.914	0.837–1.015	0.067	5.89	5.50–6.20	0.194	−0.709	±0.086
Average	15	0.891	0.809–0.994	0.068	5.88	5.56–6.11	0.154	−0.666	±0.096
25 gm. galactose and 25 gm. glucose									
Mar. 4, 1935	15	0.890	0.843–1.000	0.054	5.76	5.51–5.95	0.121	−0.627	±0.106
Mar. 13, 1935	15	0.870	0.796–0.983	0.062	6.16	5.89–6.34	0.096	−0.115	±0.172
Mar. 18, 1935	15	0.870	0.810–0.960	0.050	5.88	5.66–6.21	0.115	−0.287	±0.160
Apr. 1, 1935	15	0.880	0.801–0.989	0.060	6.14	5.87–6.41	0.174	−0.694	±0.090
May 1, 1935	15	0.878	0.788–0.993	0.056	5.94	5.79–6.07	0.082	−0.396	±0.147
Average	15	0.878	0.808–0.985	0.056	5.98	5.74–6.20	0.118	−0.424	±0.135
50 gm. lactose									
Dec. 7, 1934	15	0.836	0.765–0.922	0.048	6.17	5.98–6.39	0.112	−0.384	±0.148
Dec. 24, 1934	15	0.871	0.802–0.977	0.052	6.33	6.11–6.47	0.099	−0.555	±0.120
Jan. 2, 1935	15	0.889	0.814–0.981	0.053	6.00	5.77–6.13	0.092	−0.102	±0.172
Feb. 13, 1935	15	0.880	0.816–0.979	0.054	6.11	5.92–6.33	0.113	+0.028	±0.174
Feb. 18, 1935	15	0.880	0.808–0.973	0.055	5.92	5.62–6.03	0.116	−0.162	±0.170
Average	15	0.871	0.801–0.966	0.052	6.11	5.88–6.27	0.106	−0.235	±0.157

in each experiment for the five series. The table includes the pre-ingestion periods as well as those after the ingestion of the dose.

Control series. The R.Q.'s and the alveolar CO_2 percentages for the experiments with 250 cc. of water are plotted in figure 1. The average R.Q. after the ingestion of water, shown in solid line, was slightly lower than in the base-line periods, although there was no general trend toward either a
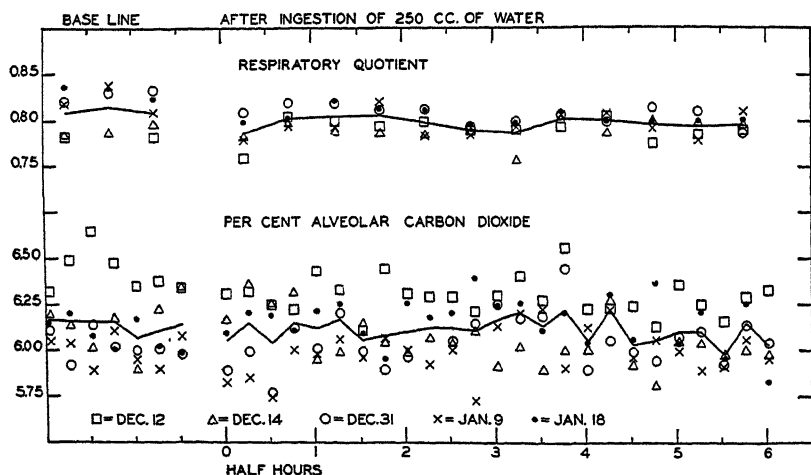
Fig. 1 Respiratory quotients and the percentages of alveolar carbon dioxide before and after the ingestion of 250 cc. of water. The respiratory quotients are plotted at the middle of each period and the carbon dioxide percentages at the points of time at which the samples were taken. The solid lines are the averages of all the experiments.

rise or a fall during the 3 hours. In general, when the base-line quotients were above or below the average line, those after taking water were, for the most part, in the same direction for a particular experiment. This is shown, for example, in the experiments of December 12th and January 9th. The range in R.Q.'s for each experiment is shown in table 1. The standard deviation shows that over two-thirds of the periods differed from the average by less than 0.02. The percentages of alveolar CO_2 show rather wide variations from the average line in figure 1. Part of this is due to the general level of

CO_2 percentages for the day. For example, table 1 shows that the average for December 12th was 6.31%, whereas for January 9th it was 5.99. The maximum range in single determinations was 0.67% on December 31st and the minimum range was 0.50% on January 9th. These ranges are wider than those given in table 1 because in table 1 the ranges are for the averages for the periods whereas figure 1 shows the ranges for the single determinations. There is no valid reason for excluding any single determination shown in figures 1 to 5 and all of the values have been used both for R.Q. and for alveolar CO_2 percentages in making up table 1. The correlation coefficients between the R.Q. and the alveolar CO_2 for the water series are low and without any definite trend toward either the positive or negative side. This finding is not illogical to expect as the measurements were really the slight variations in two physiological functions that were determined when the conditions were such that no marked trends were found. Therefore it is not to be expected that when one of these was slightly below the average the other would also vary in identically the same direction.

Twenty-five grams galactose. The results of the experiments with 25 gm. of galactose are shown in figure 2. Coincident with a marked rise in the R.Q. during the first three quarter-hours was a definite and gradual fall in the alveolar CO_2. This fall continued until just after the R.Q. started to fall. The R.Q. fell to the pre-ingestion level during the fourth half-hour. At the same time there was a rise in the alveolar CO_2. During the third hour the R.Q. tended to fall below the pre-ingestion level and at the same time the alveolar CO_2 was nearly at the pre-ingestion level. In table 1 the correlation coefficients between the alveolar CO_2 and the R.Q. for this series were consistently negative. Three of them were — 0.7 and two nearly — 0.5. Therefore as the R.Q. rose the alveolar CO_2 fell and vice versa. The greater variations in the R.Q.'s with these experiments than with the control experiments are seen in the ranges and the standard deviations for the R.Q.'s. Similarly the greater variations in the alveolar CO_2 are seen in the standard deviations.

Fifty grams galactose. The results of the five experiments with 50 gm. of galactose are shown in figure 3. A marked rise in the R.Q. was found in the first hour and was accompanied by a marked fall in the alveolar CO_2. There was but a slight fall in the R.Q. during the second hour during which time the alveolar CO_2 rose slightly but fell again to the same low level that was found during the second half-hour. From
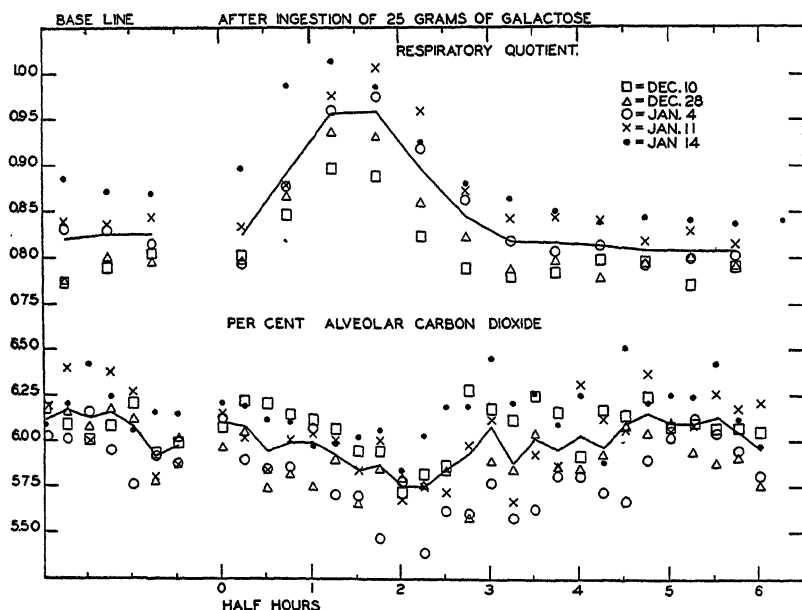
Fig. 2 Respiratory quotients and percentages of alveolar carbon dioxide before and after the ingestion of 25 gm. of galactose in 250 cc. of water. The method of plotting is the same as in figure 1.

the latter part of the third half-hour to the end of the experiment there was a gradual fall in the R.Q. to the pre-ingestion level, which was accompanied by a rise in the alveolar CO_2 to the level at the beginning of the experiment. The correlation coefficients between the R.Q. and the alveolar CO_2 for this series in table 1 are very high and negative. All of them are over -0.6 and two of them are over -0.7. Therefore there was an even more marked relationship between the

changes in the R.Q. and the changes in alveolar CO_2 with 50 gm. of galactose than there was with 25 gm. of galactose.

Twenty-five grams galactose and 25 gm. glucose. The results of the five experiments with 25 gm. of galactose and 25 gm. of glucose (the products of hydrolysis of 50 gm. of lactose) are shown in figure 4. The R.Q. had nearly the same sort of rise as was found with 25 gm. and with 50 gm. of
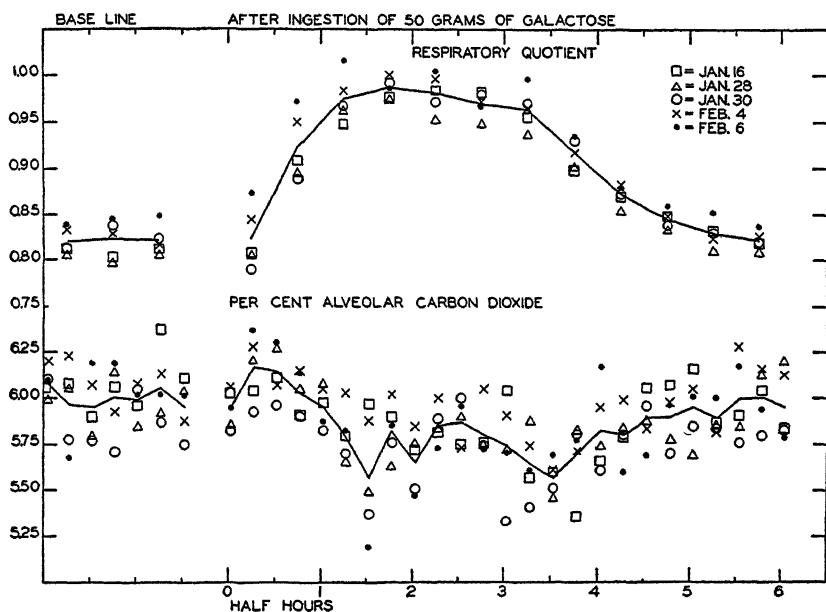
Fig. 3 Respiratory quotients and percentages of alveolar carbon dioxide before and after the ingestion of 50 gm. of galactose in 250 cc. of water. The method of plotting is the same as in figure 1.

galactose. The maximum was reached at the end of the second half-hour, but from there on the fall was more gradual during the next 2 hours than was found for those 2 hours with 25 gm. of galactose, and with 50 gm. of galactose when the fall in R.Q. began. The characteristic curve of the R.Q. after glucose (Carpenter and Fox, '30) is a somewhat more delayed maximum rise and a longer holding up of the curve than with either fructose or galactose. This effect is shown

in this series where the sugars are combined, in that the fall was not so sharp as with galactose alone. There was a tendency for the alveolar CO_2 to fall slightly and there was a generally lower level after the first half-hour until the end of the fifth half-hour. The individual determinations in this series were much more scattered than in any of the preceding series. Part of this is due to the wider range in the alveolar
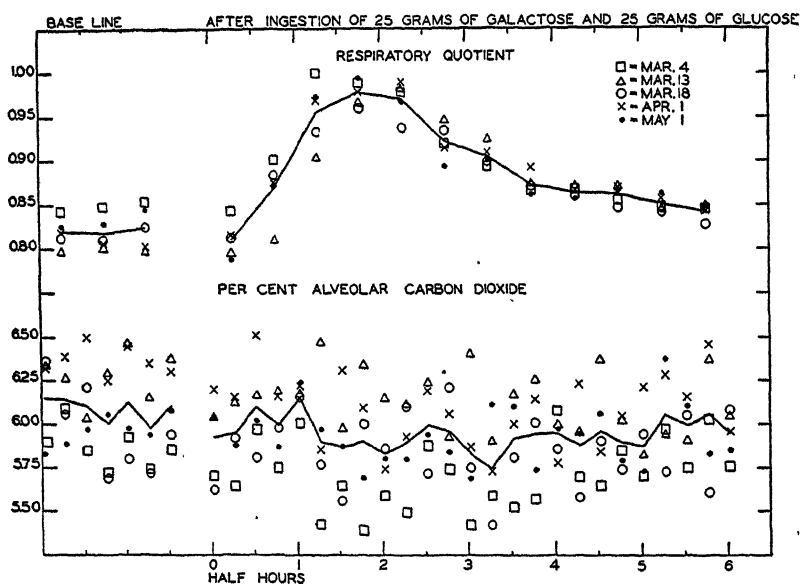
Fig. 4   Respiratory quotients and percentages of alveolar carbon dioxide before and after the ingestion of 25 gm. of galactose and 25 gm. of glucose in 250 cc. of water. The method of plotting is the same as in figure 1.

air level. The averages for this series in table 1 range from 5.76 to 6.16%, but even taking this into account, there is still a great variability in the determinations from one point to another. However, the standard deviations for the alveolar CO_2 in table 1 are not so high as with 25 gm. and 50 gm. each of galactose. Although there is a trend to a lower alveolar CO_2, it is doubtful whether a correlation can be found between the changes in the alveolar CO_2 and the changes in the R.Q., and mathematically this is evident from the variability in the

correlation coefficients in table 1. Two, namely, — 0.627 on March 4th and — 0.694 on April 1st, may be considered significant. Those on March 13th and 18th cannot be considered so and it may be a question as to whether the one on May 1st is significant. Therefore there is a great variability in the response of the alveolar CO_2 to the ingestion of these two sugars given together. The presence of glucose modifies in some way the action of galactose. This finding is in agreement with previous work on the effect of combining glucose with galactose. Folin and Berglund ('22) found that the addition of 100 gm. of glucose reduced the sugar excretion markedly in comparison with that found when galactose was taken alone. Similar results were found by Bodansky with dogs ('32), and Harding and Grant ('33) found a marked drop in galactosuria when 30 gm. of glucose were added to a dose of 40 gm. of galactose. Cori ('26) found that the rate of absorption of galactose from the intestine in rats was markedly reduced when glucose was present and Cori and Cori ('28) found there was a decrease in the galactosuria when glucose was ingested simultaneously. Whether the effect in experiments here reported is due to the slower rate of absorption when glucose is present cannot be stated. It is difficult to see how the presence of glucose and its absorption could affect the action of galactose in the formation of organic acids in its intermediary metabolism.

Fifty grams lactose. The results of the series of five experiments with the ingestion of 50 gm. of lactose are shown in figure 5. The rise in R.Q. was not so great with the lactose as with the mixture of its two components, 25 gm. of galactose and 25 gm. of glucose, and the fall is slightly more delayed. Even at the end of the experiment the R.Q. has not reached the pre-ingestion level. In this regard the course of the R.Q. differs from all of the preceding series with the sugars. A probable explanation is that there is a slight delay in the effect of the sugars due to the time required for hydrolysis of lactose. This conforms with the earlier work in this laboratory by Carpenter and Lee ('32 c). It also probably accounts for

the fact that the rise in R.Q. is not so great with lactose as with its equivalent hydrolysis products, namely, 25 gm. of galactose and 25 gm. of glucose. The alveolar CO_2 during the first hour was slightly above the pre-ingestion level, but during the third half-hour there was a definite fall. This is the period of time that corresponds to the beginning of the fall in the R.Q. Therefore this series differs from the two
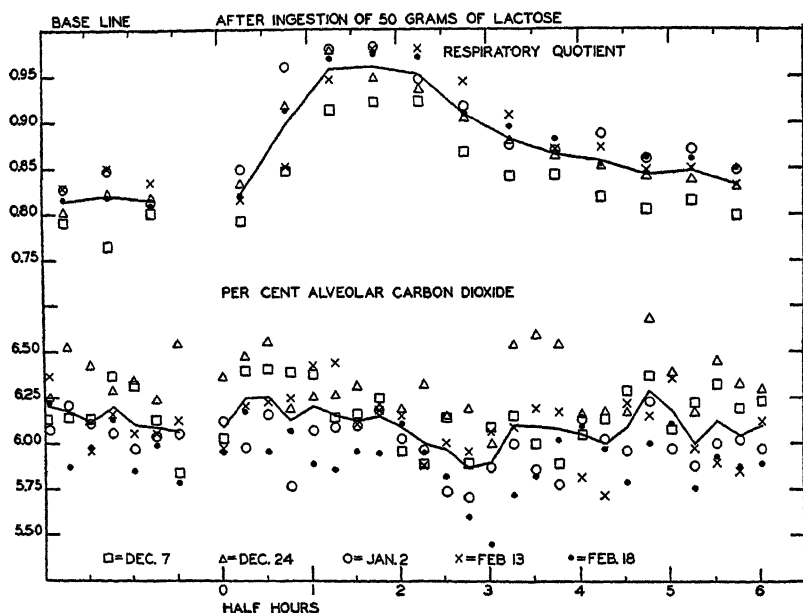
Fig. 5   Respiratory quotients and percentages of alveolar carbon dioxide before and after the ingestion of 50 gm. of lactose in 250 cc. of water. The method of plotting is the same as in figure 1.

preceding series with galactose alone in that a fall in alveolar air does not correspond to the rise in the quotient. Here again the delay may be due to the time required for hydrolysis so that galactose is not set free until later in the experiment than would be found when galactose was given alone or in combination with glucose. After the third half-hour there were irregularities in the course of the alveolar air and in general throughout the whole series. The percentages from one point to another vary considerably. However, whenever

any one group is below the average line it is so for nearly the entire experiment. This is represented in the experiment of February 18th and similarly in the experiment of December, 24th. The entire absence of the relation between the R.Q. and alveolar CO_2 in the experiments with lactose is shown in the very low correlation coefficients in table 1. Unlike all the preceding series, with the exception of the control, there is a correlation coefficient with a plus sign as well as others with negative signs. In other words, the coefficients are not consistent among themselves. The coefficients for this series are even less significant than for the preceding series with the equivalent hydrolysis products of 25 gm. each of galactose and glucose.

The preceding series of experiments show that part, at least, of the rise in the R.Q. after the ingestion of galactose must be related to the simultaneous fall in the alveolar carbon dioxide, and that this occurrence is modified by the simultaneous ingestion of another sugar, glucose. Presumably the lowering of the alveolar carbon dioxide is due to the formation of organic acids in the intermediary metabolism of galactose. Judging from previous work on the metabolism of sugars, the acid would be lactic acid. It would have been desirable to determine the lactic acid content of the blood. This, however, was not feasible in these series, and therefore the change in R.Q. due to lactic acid formation cannot be calculated. In an earlier series (Carpenter and Lee, '33 b) with this same subject, the ingestion of glucose or of fructose was not followed by any change in alveolar carbon dioxide, nor was there any change in no-dose control experiments with J.C. (Carpenter and Lee, '33 a). In the latter study, however, there was found with another subject both a lowering of the alveolar carbon dioxide and a rise in the R.Q. in the course of several hours in the no-dose control experiments. An assessment of the parallel changes (Carpenter and Lee, '33 b) in the two physiological factors with that subject showed consistently that for

each 0.10% fall in the alveolar carbon dioxide there was a rise of 0.01 in the R.Q. The application of this method of calculation of the correction of the R.Q. in these series with galactose will result in a lowering of the respiratory quotient of 0.035 in the fifth quarter-hour after the ingestion of 25 gm. of galactose as the average alveolar carbon dioxide was 5.75% in comparison with an average of 6.10% in the pre-ingestion periods. The average R.Q. at this period was 0.90, but in the period preceding this, it was 0.96, so that the maximum fall in alveolar carbon dioxide occurred in the period following the maximum rise in the R.Q. Using the same method of calculation for the experiments with 50 gm. of galactose will result in a maximum lowering of only about 0.030 in the R.Q. in the fourth and seventh quarter-hours after the sugar was given. The average alveolar carbon dioxide was 5.71 and 5.69% in these two periods in comparison with an average of 6.00% in the pre-ingestion periods. The respiratory quotients averaged 0.99 and 0.96 in these two periods, respectively. This method of calculation cannot be applied satisfactorily to the group with 25 gm. each of galactose and glucose combined because the maximum R.Q. occurred in the fourth and fifth quarter-hours after the sugars were given, whereas the most marked lowering of the alveolar carbon dioxide was not until the seventh quarter-hour. In spite of the marked rise in the R.Q. after lactose, there was no significant change in the alveolar carbon dioxide in relation to the changes in the R.Q. Assuming these experiments are of significance in the interpretation of the R.Q. after the ingestion of galactose, the indications are that although organic acids are formed in the intermediary metabolism of galactose, the amounts are not large enough to change very materially the characteristic form of the curve of the R.Q. after the ingestion of this sugar. The amounts formed probably are large enough to explain the occurrence of an R.Q. over unity, but not enough to cause the course of the R.Q. to closely resemble that after the ingestion of glucose.

EXCRETION OF SUGAR IN THE URINE

As a further contribution to the question of the tolerance of galactose the urines were collected and analyzed in these experimental series for the period of time beginning with the arrival of the subject in the morning and ending immediately after the last respiratory exchange measurement. This period therefore included the time during the preliminary rest and the first three quarter-hours of respiratory exchange measurements in addition to the periods after the ingestion of the dose. The average total time covered was about 5 hours. The sugar (reducing substances) was determined by the method of Folin ('26) with the modifications of Folin and

TABLE 2

The excretion of sugar[1] in the urine as affected by the ingestion of galactose, galactose and glucose, and lactose with J.C. (approximately 5 hours)

250 CC. WATER	25 GM. GALACTOSE	50 GM. GALACTOSE	25 GM. GALACTOSE + 25 GM. GLUCOSE	50 GM. LACTOSE
gm.	*gm.*	*gm.*	*gm.*	*gm.*
0.09	0.73	2.11	0.20	0.36
0.08	0.61	1.85	0.21	0.16
0.10	0.38	2.03	0.21	0.17
0.10	0.35	1.53	0.19	0.18
0.08	0.70	1.55	0.72	0.37

[1] Calculated as glucose.

Svedberg ('26). The results are shown in table 2. The average excretion in the control series was only 0.09 gm. calculated as glucose and was very uniform for the series. With 25 and 50 gm. of galactose, there was an average excretion of 0.55 and 1.81 gm. as glucose. Calculated as galactose, the values would be 1.19 and 3.93 gm., respectively. Thus there was a definite and appreciable galactosuria with 25 and 50 gm. of galactose. The same subject in a previous study with galactose (Carpenter and Lee, '32) gave an excretion in about 4½ hours of 0.7, 1.0, 2.2 and 3.1 gm. as galactose with an ingestion of 10, 20, 30 and 40 gm. of galactose, respectively. When 50 gm. of lactose or 25 gm. of galactose and of glucose together were ingested, the average sugar excretion as glucose

was only 0.25 and 0.31 gm. Hydrolysis of the urines after lactose ingestion did not increase significantly the amount of reducing substances. Four of the five experiments with combined galactose and glucose averaged only 0.20 gm. These values are lower than those found with 25 gm. of galactose alone. This is in line with earlier findings on the effect of glucose on excretion of galactose (see p. 591).

TABLE 3

Average changes from the base line in nutrients catabolized and heat production with J.C. after the ingestion of galactose, galactose and glucose, and lactose (3-hour period)

KIND OF EXPERIMENT	CARBOHYDRATES	FAT	HEAT PRODUCTION	
			Average base line	Average change
	gm.	*gm.*	*calories*	*calories*
Control, 250 cc. water	— 2.2±2.4	+0.9±0.9	199	—0.5±1.4
25 gm. galactose	+ 5.5±0.7	—2.2±0.7	197	+1.9±4.0
50 gm. galactose	+15.3±0.5	—5.8±0.3	190	+8.8±3.4
25 gm. galactose + 25 gm. glucose	+12.4±1.7	—5.0±1.2	196	+4.7±4.4
50 gm. lactose	+11.5±2.3	—4.3±1.1	197	+7.0±0.9

METABOLISM OF NUTRIENTS AND HEAT PRODUCTION

Although this investigation was not made to determine particularly the effect of the ingestion of galactose on the catabolism of nutrients, a summary is given in table 3 of the changes in catabolism of carbohydrates and fat, and in the heat production. The calculations have been made in the conventional manner of correcting for the protein on the assumption that the nitrogen excreted in the urine represents the protein catabolized during the period of urine collection. It has also been assumed that the R.Q. was a true metabolic quotient, that is, the result of complete combustion of carbohydrate and fat, and protein except for the residue excreted in the urine. This may seem inconsistent with the finding of a change in the alveolar CO_2, but presumably there is a compensation for the loss in CO_2 so that the R.Q. for the 3 hours

represents the net sums of the total respiratory exchange. Table 3 shows the mean for each series of experiments with the average deviation of the individual experiments from the mean for the group.

The control series does not show any definite effect on either the catabolism of nutrients or the heat production as the average changes are too small and the average deviations too large for the changes to be of significance.

The ingestion of 25 and 50 gm. each of galactose caused increases of 5.5 and 15.3 gm. of carbohydrate catabolized. Earlier studies (Carpenter and Lee, '32 a) gave with this same subject for a 2½-hour period with 20, 30 and 40 gm. of galactose 3.9, 7.5 and 12.8 gm. increase in carbohydrate catabolized. The results in the series here reported are consistent with the earlier studies. The combination of 25 gm. each of galactose and glucose caused an increase of 12.4 gm. of carbohydrate catabolized. An earlier study with this same subject (Carpenter and Lee, '32 c) with 20 gm. each of galactose and glucose resulted in an average increase of 9.2 gm. in 2½ hours. The ingestion of 50 gm. of lactose caused an increase of 11.5 gm. of carbohydrate catabolized in comparison with 12.4 gm. when the equivalent products of hydrolysis were given and 9.3 gm. in the earlier study in 2½ hours after the ingestion of 40 gm. of lactose.

Not only was there an increase in the carbohydrate catabolized, but also a decrease in catabolism of fat. The decrease with 25 gm. of galactose was only 2.2 gm., but it is definite as all five experiments showed a decrease. The decreases were more marked with the other three series of experiments ranging from 4.3 to 5.8 gm. and all of the individual experiments showed decreases.

The average increase in heat production was only 1.9 calories with 25 gm. of galactose and the variability is so large that this increase cannot be considered significant. The increase with 50 gm. averaged 8.8 calories. This value probably does not represent the true increase as there was an increase of only 0.5 calorie in one experiment, and the other four gave

increases ranging from 9.5 to 12.5 with an average of 10.9 calories. The increase in heat production after the combination 25 gm. each of galactose and glucose averaged only 4.7 calories with variations ranging from — 1.0 to + 11.8 calories. The increase with 50 gm. of lactose was more definite at 7.0 calories with the increases ranging from 5.8 to 8.6 calories. In agreement with earlier experience in this laboratory, there was not so good a uniformity in the changes in heat production after the ingestion of sugars as there was in the effect on the catabolism of carbohydrates.

SUMMARY

The respiratory quotient was determined with a human subject in 15-minute periods before and for 3 hours after the ingestion of 250 cc. of water as a control, 25 and 50 gm. each of galactose, 25 gm. of galactose plus 25 gm. of glucose, and 50 gm. of lactose, in 250 cc. of water. Alveolar carbon dioxide was determined simultaneously at 7- to 8-minute intervals during the periods of respiratory exchange. There were five experiments in each group

The ingestion of 25 and of 50 gm. of galactose was accompanied by marked drops in the alveolar carbon dioxide that lasted over 1 hour with 25 gm. and over $1\frac{3}{4}$ hours with 50 gm. of galactose. In the same periods of time there were marked rises in the respiratory quotients. The correlation coefficients between the alveolar carbon dioxide and the respiratory quotients in the two groups averaged — 0.640 and — 0.666.

With 25 gm. of galactose and of glucose together there was a drop in alveolar carbon dioxide and a rise in respiratory quotient, but the changes in the two factors were not synchronous and the correlation coefficients between the two factors averaged only — 0.424 with a P.E.$_r$ of ± 0.135.

After 50 gm. of lactose there was a fall in the alveolar carbon dioxide for $1\frac{1}{2}$ hours, but the maximum respiratory quotient came earlier, in the third quarter-hour, so that the correlation coefficient between the two factors was only — 0.235. Evidently there was a latency in the effect on the

alveolar air due to the time required for the hydrolysis of the lactose.

Although organic acids were not determined in the blood or urine, the falls in alveolar carbon dioxide accompanying the rises in the respiratory quotient after the ingestion of galactose indicate the formation of acid products in the intermediary metabolism of galactose.

The ascending order of elimination of reducing substances (sugar) in the urine was: control series, 50 gm. lactose, 25 gm. each of glucose and galactose, 25 gm. of galactose, and 50 gm. of galactose.

The increases in the catabolism of carbohydrates and in the heat production in these series agreed in the main with similar series previously carried through in this laboratory.

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EDITORIAL REVIEW /

THE NUTRITIONAL AND METABOLIC SIGNIFICANCE OF CERTAIN ORGANIC ACIDS

ARTHUR H. SMITH AND JAMES M. ORTEN ·

Yale University School of Medicine

The organic acids are widely distributed in many common foods. Citric, malic, tartaric, oxalic and benzoic acids, for example, are present in numerous fruits and vegetables; other less common acids, such as succinic, isocitric, aconitic, tricarballylic, malonic, glyoxylic, salicylic and quinic acids, also occur in some foods. Likewise, the presence of a number of the organic acids in mammalian tissues and body fluids has been repeatedly demonstrated. Questions therefore arise regarding the origin, possible effects, and the ultimate fate of these substances in the organism. Obviously, they may be derived from the preformed acids in ingested food. However, there is convincing evidence that certain acids are endogenous in origin and that they may be formed in the course of the metabolism of the carbohydrates, fats, or proteins. Also, there are increasing indications that certain organic acids are not fortuitous constituents of living matter but that they are involved in definite physiological processes. Although there are other directions in which interest in the organic acids has been focused, the present review must of necessity limit its scope to the more general problems cited above, namely, the occurrence, origin, possible functional significance, and metabolic fate of these compounds. The nutritional and metabolic behavior of 'total' organic acids, as present in certain foods, will be considered first and will be followed by a discussion of several of the more widely studied individual acids known to occur in edible substances.

TOTAL ORGANIC ACIDS

The fact that certain foods upon combustion yield an alkaline ash has long been known. This relationship was determined quantitatively in the comprehensive studies of Sherman and Gettler ('12); the preponderance of base-forming elements was particularly striking in the case of fruits and vegetables. Many of the foods which yielded an alkaline ash on combustion likewise exerted an alkalinizing effect, as indicated by an increase in the pH and a decrease in the titratable acidity of the urine, when ingested by human subjects (Blatherwick, '14). A consistent exception to this general relationship was noted, however, in the case of prunes, plums and cranberries which, although forming an alkaline ash, decreased the pH and increased the titratable acidity of the urine. At that time, the explanation of 'potential alkalinity' of foods offered was that some base was present in the foods combined in the form of organic acid salts, and that during the process of metabolism the organic acid ion was oxidized, largely to bicarbonate. The different behavior of the substances mentioned above was attributed to the presence in these foods of relatively large amounts of benzoic acid, a substance not oxidized in the organism but excreted as hippuric acid.

Satisfactory evidence for the foregoing explanations of the 'potential alkalinity' of foods was lacking until a reliable method for the quantitative determination of organic acids in biological materials was developed. The introduction of the method of Van Slyke and Palmer ('20) for estimating organic acids in urine marked the beginning of the period of significant progress. This method is based on the principle that, after the preliminary removal of phosphates and carbonates with calcium hydroxide, the organic acids of urine may be determined by titration from a pH of 8.0 to 2.7, using suitable indicators. If protein is present, it should be removed before the sample is titrated. Since creatinine, creatine and amino acids are also titrated in this pH range, corrections should be made for these substances, particularly creatinine. The

fact that oxalic, some citric, and perhaps other organic acids are undoubtedly lost during the treatment with calcium hydroxide, appears to be a valid criticism of the Van Slyke and Palmer method. There have appeared numerous modifications of the original method which for the most part involve the extraction of the organic acids from acidified urine prior to titration, changes in the preliminary treatment of the samples to remove interfering substances, or the use of other indicators. Determinations made by the foregoing methods show that the amount of organic acid excreted daily by the normal adult is approximately 6 cc. N/10 acid per kilogram body weight (corrected for creatinine). Methods have also been described for the determination of organic acids in blood by indicator titration, by electrometric titration, and by calculation (subtracting the sum of the anions bicarbonate, chloride, phosphate, sulfate, and the base-combining power of proteins from values for total base of whole blood or plasma). Amounts of organic acids equivalent to 10 to 20 millimols of univalent base per 100 cc. are present in the blood serum of normal adult human subjects.

By means of the foregoing methods, considerable information regarding the fate of ingested organic acids and the behavior of these substances in various physiological and pathological conditions has been obtained. In some of the earlier studies (Blatherwick and Long, '22, '23; Chaney and Blunt, '25), as well as in later ones (Schuck, '34 a), it was shown that the administration to human subjects of organic acids in the form of orange juice led to the excretion of a distinctly alkaline urine and to only a relatively small increase in the amount of organic acids eliminated. These studies demonstrate convincingly the fact that the salts of organic acids as they occur in certain fruit juices yield basic substances in the organism which are excreted in the urine. The striking capacity of the organism to metabolize the organic acids of orange juice was also brought out clearly in studies showing that the acids of as much as 2400 cc. daily of orange juice were almost completely destroyed. Similar

experiments with sour milk showed a comparable utilization of its organic acids. In other studies, however, it was found that the organic acids present in certain other fruits behaved differently than those of orange juice (Blatherwick, '14; Blatherwick and Long, '23; Fellers, Redmon and Parrott, '33). For example, the ingestion of prunes, plums and cranberries was followed by the excretion of a more acid urine. This was attributed to the inability of the organism to oxidize certain aromatic acids present in these fruits.

Likewise, it has been shown that variations occur in the metabolism of the organic acids of other fruits and foods. These studies have included grapes (Pickens and Hetler, '30; Saywell, '32 a; Pratt and Swartout, '33 a; Schuck, '34 a; Clouse, '35), figs and raisins (Saywell, '32 b), tomatoes (Saywell and Lane, '33), pineapples (Miller, '28; Clouse, '35), and loganberries, grapefruit, lemons, apples and sauerkraut (Clouse, '35). Although there is some variation between the results obtained by different investigators, it appears that the juices of the orange and tomato increase the pH of the urine more than do equal amounts of grapes, grapefruit, apples and lemons. However, even in the case of the grape itself, decided variations in response have been observed. This is probably due in part at least to differences in the varieties of grapes used (Saywell, '32 a) and in part perhaps to differences in the amounts employed by various investigators. The fact that grapes increase the pH of urine less than equal amounts of oranges, for example, may be due to the fact that grapes contain a rather large amount of tartaric acid, a compound difficultly oxidized in the animal body.

The question of variations in the total organic acid content of the blood following the ingestion of foods rich in organic acids does not appear to have been studied in detail. It may be pointed out in this connection, however, that no consistent significant alteration in the 'alkali reserve' (plasma CO_2) of the blood has been observed in human subjects following the ingestion of relatively large amounts of organic acids in the form of oranges, prunes and cranberries (Fellers, Redmon

and Parrott, '33; Bischoff, Sansum, Long and Dewar, '34). Few metabolic studies have been made on the organic acids of feces; the presence in the intestine of bacteria, which themselves are known to destroy and to synthesize certain organic acids, makes an interpretation of such studies difficult.

That the organic acids appearing in the urine may not necessarily represent those which have escaped oxidation in the organism is suggested by the work of several investigators (see Schuck, '34 a). For example, the fact that high protein diets augment the excretion of organic acids in the urine has been repeatedly shown (see McLaughlin and Blunt, '23). However, it should be borne in mind that as yet the identity of these acids has not been determined. Likewise, there is other evidence that internal factors may influence organic acid excretion in the urine. Among these may be cited the increased excretion in infant's urine during undernutrition (Utheim, '21), the marked increase found during alkalosis (Goiffon, '25; Fasold, '31), and the decrease following the administration of acids (Fasold, '31). The latter findings have led to the formulation of an hypothesis (Fasold, '31) that organic acids play an important role in acid-base balance regulation. Such factors as exercise, hyperventilation and anoxemia are also known to increase the organic acid content of the blood (Peters, Bulger, Eisenman and Lee, '26) and urine (lactic acid) (Liljestrand and Wilson, '24). Increases in the organic acid content of blood and of urine have been described in pneumonia, diabetes, nephritis, and other pathological conditions. The elevated values in diabetes appear to be due largely to the presence of 'acetone bodies'; the identity of the substances responsible for the rises in the other pathological conditions, however, is still unknown.

Thus it is evident from studies on 'total' organic acids that there are obvious differences in the nutritional significance and in the pathways of metabolism of the various single acids; it now becomes essential to study the behavior of the individual acids themselves.

TARTARIC ACID

Tartaric acid was first prepared as the free acid from 'weinstein' (potassium acid tartrate) by Scheele in 1770 (see Retzius, 1770) by a method described in his first published work. The potassium acid tartrate was converted to the insoluble calcium salt by chalk and free tartaric acid was then produced by treatment with sulfuric acid. Crystalline tartaric acid was prepared by the evaporation of the clear filtrate.

The presence of tartaric acid in a number of common foods is now well established. Of the methods described for the quantitative determination of the acid in biological materials, the potassium acid tartrate and the Kling procedures have been most widely used; they have been tentatively adopted by the American Association of Official Agricultural Chemists. The former method depends on the preliminary precipitation of tartrate as the lead salt, subsequent conversion to and isolation as potassium acid tartrate, and finally titration of this salt with standard alkali. The Kling procedure depends on the permanganate titration of tartrate isolated as calcium racemate, after preliminary precipitation as the lead salt. More recently, a colorimetric method, depending on the formation of a deep red color with meta-vanadate in the presence of tartrate, has been described (Underhill, Peterman and Krause, '31). Under certain conditions the method is said to be specific and reliable. Thus far it has been applied only to the determination of tartrates in urine and other biological material.

Analyses of a few common foods, using the potassium acid tartrate method, have been reported recently (Hartmann and Hillig, '34). Of some twenty-nine fruits and twenty-nine vegetables studied, only the following contained quantities of tartaric acid exceeding 0.014%, an amount believed to be attributable to foreign acid-reacting substances in the cream of tartar precipitate: grape juice, 1.07%; dried lentils, 0.19%; black raspberry, 0.028%; artichoke, 0.020%; avocado, 0.020%; and quince, 0.018%. The outstanding point brought out by analyses thus far reported is the fact that, with the

exception of the grape, only small amounts of tartaric acid are present in the common foods examined.

The question of the fate of tartaric acid entering the organism has been studied by a number of investigators with somewhat conflicting results. This appears to be due, in part at least, both to fallacies in the methods employed and to species differences in the metabolism of the compound. The consistent findings of the early investigators (see Finkle, '33) that only 10 to 40% of tartaric acid administered orally (as the sodium salt, usually) was recovered in the urine, was interpreted as indicating that this acid is largely oxidized in the animal organism. However, subsequent studies in which tartrate has been administered parenterally to different species, including man (Simpson, '25; Underhill, Leonard, Gross and Jaleski, '31; Underhill, Peterman, Jaleski and Leonard, '31; Finkle, '33), have shown conclusively that this belief is entirely erroneous, since 90% or more of the injected compound can be recovered in the urine. Indeed, according to one report (Underhill, Leonard, Gross and Jaleski, '31) even orally administered tartrate is excreted almost quantitatively in the urine in the case of the rabbit, dog and rat.

That the foregoing apparent discrepancy in results is due largely to species differences in the metabolism of tartaric acid is now recognized. In contrast to the above findings in the rabbit, dog and rat, only 20% or less of the tartrate administered orally to guinea pigs and to man was eliminated in the urine (Underhill, Leonard, Gross and Jaleski, '31; Underhill, Peterman, Jaleski and Leonard, '31). No extra tartaric acid appeared in the feces. Three possible explanations for these findings may be advanced: that the ingested tartrate was not absorbed and was destroyed by bacteria or by digestive secretions in the gastrointestinal tract; that it was absorbed and stored in the tissues; that it was oxidized in the organism. It appears unlikely that either of the last two hypotheses is tenable in view of the fact that appreciable storage of tartrate administered parenterally to these species does not occur and that respiratory metabolism studies

(Underhill, Peterman, Jaleski and Leonard, '31) give no indication that oral tartrate is oxidized either by the guinea pig or by man. On the other hand, the evidence appears convincing that considerable amounts of tartrate may be destroyed in the intestinal tract of the rabbit and of man by the action of bacteria (Simpson, '25; Underhill, Peterman, Jaleski and Leonard, '31; Pratt and Swartout, '33 b). That the decomposition is brought about by the action of bacteria rather than by that of some intestinal secretion is indicated by the fact that bile, pancreatin, the contents of the small intestine and Berkefeldt filtrates of fecal material (Underhill, Peterman, Jaleski and Leonard, '31; Pratt and Swartout, '33 b) exert little or no effect on tartrates, whereas fecal material or cultures of fecal organisms alone produce a rapid and complete destruction of the salt. These investigations also obviously indicate that the decomposition of tartrates occurs in the large intestine.

The general observation that foods rich in tartrates, as grapes and grape products, exert a mild 'alkalinizing effect' when ingested in sufficient amounts was discussed earlier. This has been attributed by one group of investigators (Pratt and Swartout, '33 b) to the absorption from the intestine of alkaline decomposition products of tartrates. Possible support for this explanation is given by the fact that the medium in which tartrate had been decomposed by cultures of fecal bacteria became distinctly alkaline. There remains, however, the possibility that the potential alkalinity of grapes may be due to the presence in the grape of substances other than tartrates.

The fact that tartrates may produce nephritis characterized by marked degenerative changes in the renal tubules, regardless of the mode of administration, has been adequately demonstrated in several species (see Rose, '24–'25). The point should be brought out, however, that the production of nephrotoxic effects requires the administration of amounts of tartrate above a fairly definite critical level. In the dog (Underhill, Leonard, Gross and Jaleski, '31) this level appears to be at least 400 mg. of tartaric acid per kilogram body

weight, administered orally. No detectable renal damage occurred in dogs fed smaller amounts than this. Changes in the character of the diet (low calcium, high calcium, high carbohydrate, and high fat) or fasting did not appear to appreciably influence the nephrotoxic effect of tartrate in dogs. According to the same investigators, the rabbit appears to be somewhat more susceptible to the nephrotoxic effects of tartrate than is the dog, doses of 100 mg. per kilogram body weight producing renal damage. While it is recognized that the foregoing amounts required to produce renal damage in experimental animals are far in excess of those which could possibly be ingested in even large quantities of food relatively rich in tartrates, these findings nevertheless indicate the necessity of caution in using tartrates in large or repeated amounts.

MALIC ACID

Another of the organic acids of importance from the point of view of nutrition is malic acid. The ubiquitous distribution of this acid and its salts in nature, the ease of production in large quantities and the promise of greater usefulness in food processing have increased the attention devoted to this substance. Furthermore, the newer studies on intermediary metabolism carried out with isolated tissue indicate that malic acid plays a part in the chemical changes taking place in the body. Scheele (1785 a) discovered malic acid in the juice of unripe apples and berries. Braconnot, in 1808, obtained malic acid from the leaves of euphorbia and its presence in tobacco leaves was demonstrated by Vauquelin the following year. It was first found in the unripe berries of the mountain ash by Liebig in 1833 and for many years this was an important source of the compound.

Liebig (1833) noted the fact that the conventional methods for determining organic acids in plants could not be used for malic acid. According to Hartmann and Hillig ('32) no specific precipitant is known which can serve in the isolation and estimation of the acid. Most of the methods employed

take advantage of the fact that, when uranium acetate or nitrate is added to solutions of malic acid, the optical rotation is markedly augmented, the gross increase being proportional to the quantity of the acid present. The great difficulty has been to secure a solution of malic acid free from the many accompanying constituents of the fruit and plant juices, such as pectin, sugars and other organic acids. The pectin is removed with strong alcohol and the di- and tricarboxylic acids as well as the tannins separated from the sugars and other acids by lead acetate. Tartaric acid is removed as potassium acid tartrate and the other acids by precipitation with tribasic lead acetate. After concentrating, the filtrate is saturated with uranium acetate and the malic acid determined polarimetrically.

A large number of analyses upon fruits and plant tissue have been carried out by Franzen and his co-workers (see Franzen and Schumacher, '21) by precipitating the acids as the lead or barium salts and subsequent fractional distillation of the ethyl esters under reduced pressure. Although this method is well adapted to the identification of organic acids, the large amount of material required for the analyses is a drawback. From this point of view alone, the recent method of Pucher, Vickery and Wakeman ('34) represents a distinct advantage. In this procedure the malic acid is converted by permanganate and potassium bromide into a volatile product which is distilled with steam into diphenylhydrazine in acid solution. The insoluble condensation product, after solution in pyridine, yields a blue color with alkali, which color is proportional to the amount of malic acid originally used. Thus with samples containing as little as 0.2 mg., the recovery of malic acid varied from 96 to 106%.

The extremely wide distribution of malic acid and its salts in plants and plant products is now recognized. Thus, it is found frequently along with other acids, in sugar beets, currants, rhubarb, alfalfa and maple sap, the calcium salt being known in the latter case as 'maple sand.' Hartmann and Hillig ('34) have presented analyses for malic acid in a

large number of common fruits and vegetables. The values in fresh fruits range from 2.48% in Damson plums, 1.59% in quinces and 1.02% in crab apples to as little as 0.16% in strawberries and 0.08% in grapefruit. Of the common varieties of apples the early Yellow Transparent has 0.97% malic acid and the popular Delicious only 0.27%. The vegetables including roots, leaves, seeds and fruits are notably poor in malic acid, as 'acid' a vegetable as the tomato containing only some 0.05%. As might be expected, fruit jellies and jams contain measurable quantities of malic acid (Hartmann and Hillig, '32) as does molasses from sugar cane. According to Steuart ('35) malic acid disappears from apple cider within 10 weeks giving rise to lactic and acetic acids.

Little definite information appears in the literature regarding the specific effects of malic acid in the diet or its behavior in the animal body. The metabolism of organic food acids in general has been discussed earlier in this review; inasmuch as malic acid accounts for a large part of the total acidity in many fruits and vegetables, the discussion of the metabolism of the total organic acids probably applies fairly closely to malic acid itself. Ohta ('12) using an adaptation of the uranyl acetate-polarimetric method for the estimation of malic acid in urine, found that in both the rabbit and the dog a very large proportion of the quantity given by mouth or subcutaneously was destroyed. Sodium malate produces marked gastric irritation according to Underhill and Pack ('25). These investigators gave 1 gm. sodium acid malate per kilogram body weight to fasting dogs and noted prompt and definite reduction in the titratable acidity and a decrease of the ammonia in the urine. These responses are obviously associated with the acid-base balance and indicate that the malate was oxidized most likely to bicarbonate.

Malic acid appears to be involved in carbohydrate metabolism in the animal body as well as in plants. When the sodium salt was administered either subcutaneously or by mouth to phlorhizinized dogs, extra glucose was excreted in the urine and the D:N ratio indicated that it did not arise

from accelerated protein catabolism (Ringer, Frankel and Jonas, '13). Again Ponsford and Smedley-Maclean ('32) showed that in rats fed a carbohydrate-poor diet, ammonium malate per os produced an unmistakable increase in liver glycogen. In contrast to the decided nephrotoxicity of tartaric acid, l-malic acid is innocuous and the d-form only slightly toxic (Rose, '24–'25).

From the available evidence, it appears that, like citrates but in contrast to tartrates, malates are readily metabolized in the body; these acid salts may thus be regarded as potential alkalies in nutrition.

CITRIC ACID

The presence in certain foods of the acid now called citric acid was known to Scheele who isolated the free acid from lemon juice in 1784. The substance was prepared by precipitation of the calcium salt from hot aqueous solution and subsequent crystallization of the compound from weak sulfuric acid solution. Since then the presence of citric acid in varying amounts has been reported in numerous plant substances.

The recent expansion of knowledge regarding the behavior of citric acid in the animal organism has been a direct result of the development of reliable methods for the quantitative determination of this substance. Two general types of methods for the estimation of citric acid are now in use, the 'enzyme' method and methods based on the Stahre reaction (1895). The enzyme method (see Thunberg, '29; Östberg, '31; Kuyper and Mattill, '33) is based on the fact that the enzyme, citric acid dehydrogenase, in the presence of a hydrogen acceptor, methylene blue, catalyzes the liberation of hydrogen from citric acid at a rate proportional to the concentration of citric acid. By determining the rate of decolorization of the methylene blue under standardized conditions, it is possible to determine quantitatively amounts of citric acid as small as 0.2 mg. per 100 cc. This method has the advantages of sensitivity to extremely small amounts of citric

acid and of specificity, although isocitric acid is also dehydrogenated under the same conditions. However, the applicability of the method is limited, as are many methods of this type, because of the extreme sensitivity of the enzyme to variations in the composition of the reaction medium.

Probably the most reliable methods for the determination of citric acid at the present time are those based on the Stahre reaction. The principle of these methods is the oxidation of citric acid in the presence of bromine to an insoluble compound, pentabromacetone, which may be determined by weighing, by titration of its bromine, or by a colorimetric method in which pentabromacetone treated with sodium sulfide forms a yellow-orange colored complex. The reaction is remarkably specific, such closely related compounds as isocitric, aconitic, and tricarballylic acids failing to form pentabromacetone under the same conditions. The few known interfering substances may be removed by preliminary boiling in acid solution or by precipitation with bromine water. The final measurement of the pentabromacetone formed may be made gravimetrically if amounts of citric acid exceeding 50 mg. are present in the samples, as in the case of foods (see Hartmann and Hillig, '34). The titrimetric procedure (see Pucher, Vickery and Leavenworth, '34) may be used if the amount of citric acid in the sample is between 1 and 20 mg., and the colorimetric technic (see Pucher, Sherman and Vickery, '36) is satisfactory for the determination of amounts varying from 0.1 to 1.0 mg. The latter method has proved reliable in the hands of the present authors for the determination of the citric acid content of blood, urine and various tissues.

Using the gravimetric pentabromacetone procedure, Hartmann and Hillig ('34) have reported values for the citric acid content of a number of fruits, vegetables, and miscellaneous foods. Of the fruits, lemon juice contained the highest concentration of citric acid, 6.08%, while only small amounts were found in plums, peaches and apples. Whole oranges and grapefruit contained 0.92 and 1.33%, respectively. Of

the vegetables studied, lima beans, kale, white potatoes and tomatoes were found to contain the largest amounts of citric acid, 0.35 to 0.65%. Small quantities are found in cocoa, malt and wheat germ, whereas certain sea foods, as oysters, clams, crabs, shrimps and scallops, contain almost none. Dried brewer's yeast contains approximately 0:3% citric acid (Sherman, Mendel and Smith, '36 a).

The presence of citric acid in the animal organism was first described by Soxhlet and Henkel in 1888. The crystalline compound was isolated from cow's milk by precipitation of the calcium salt from hot deproteinized whey, and subsequent conversion of the salt to the free acid which was then extracted with ether and crystallized. Since then citric acid has been found in the milk of several species, including that of man. The amount present is surprisingly large, 1.0 to 4.0 gm. per liter (see Östberg, '31); however, colostrum apparently contains no citric acid (Jerlov, '29). The presence of this acid in normal human urine was reported first by Amberg and McClure ('17) and recently the crystalline compound has been prepared from human urine (Fasold, '30). The amount excreted daily by man varies from 0.2 to 1.0 gm. (see Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34; Pucher, Sherman and Vickery, '36). The dog excretes 5 to 20 mg. daily (Pucher, Sherman and Vickery, '36; Orten and Smith, '37) and the rat, 0.3 to 0.6 mg. (1.0 to 2.0 mg. per kilogram body weight) daily (Pucher, Sherman and Vickery, '36). Citric acid is present in the urine of the newborn infant (Lennér, '34; Boothby and Adams, '34). The presence of this substance in almost every other body fluid, and in many tissues has also been described. The following approximate amounts, expressed as milligrams per cent unless otherwise stated, have been reported: sweat, 0.1 to 0.2 (Scherstén, '36); aqueous and vitreous humor of the eye, 3.0 (Grönvald, '36); spermatic fluid, 1.0 to 4.0 (Scherstén, '36); cerebrospinal fluid, 5.0 (Thunberg, '29; Boothby and Adams, '34); saliva, 1.0 (Pucher, Sherman and Vickery, '36); whole blood, 1 to 2 (Östberg, '31; Pucher, Sherman and Vickery, '36); blood cells,

0.5 to 1.2 and blood serum, 0.7 to 1.8 (Pucher, Sherman and Vickery, '36); muscle, liver, kidney, spleen, pancreas, and tumor tissue, 0.1 to 3.0 (Pucher, Sherman and Vickery, '36); feces (dog), 0.4 to 0.8 mg. per 24 hours (Pucher, Sherman and Vickery, '36). Two important facts are brought out by these data, the presence of citric acid in all body fluids and tissues examined and the absence of stores of the substance. The significance of these observations will be discussed subsequently.

Questions logically arise regarding the origin of the citric acid found in animal body fluids and tissues. Obviously it may come from the citric acid present in ingested food. As was pointed out in the section on total organic acids, the available evidence indicates that probably 90 to 95% of the organic acids ingested in the form of orange juice are destroyed in the organism, even when relatively large amounts are consumed. Since citric acid is one of the important organic acids in the orange, these findings suggest that citric acid administered by mouth may be almost completely metabolized. Studies in which pure citric acid was given orally to human subjects have shown that this is true (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34; Schuck, '34 b; Sherman, Mendel and Smith, '36 b). In the dog, large amounts of orally administered citric acid are likewise destroyed, less than 1% of the compound given appearing in the urine and no extra citric acid appearing in the feces (Sherman, Mendel and Smith, '36 b). Similar results have been obtained in rabbits. The possibility that the administered citric acid was not absorbed appears to be precluded by the finding of a prompt and prolonged rise in the level of blood citric acid immediately after the ingestion of the compound. Likewise, the possible importance of destruction by bacteria in the intestine is minimized by the finding (Langecker, '34) that the enzymes and bacteria present in the intestinal tract of the rabbit do not destroy citric acid even after incubation for as long as 9 hours. Therefore, orally

administered citric acid is undoubtedly absorbed and metabolized by the organism. Some may be converted to carbohydrate, inasmuch as there is evidence that citric acid forms glucose in the phlorhizinized dog (Greenwald, '14). Some is probably oxidized in the organism, perhaps chiefly in the liver and in muscle, since these two tissues are known to contain citric acid dehydrogenase (see Reichel and Neeff, '36). Some support to the latter view is given by the observations that fresh liver and muscle tissue of the rabbit catalyze the in vitro destruction of citric acid (Battelli and Stern, '11; Langecker, '34). The involvement of the liver in the destruction of citric acid is further suggested by the finding of a progressive increase in the amount of the substance excreted in the urine following hepatectomy in dogs (Boothby and Adams, '34). Further indication of the marked ability of the organism to metabolize citric acid is the finding in the rabbit and dog that some 60% of the sodium citrate injected intravenously does not appear in the urine (Salant and Wise, '16–'17; Orten and Smith, '37).

If it is true that citric acid ingested in foods is almost completely destroyed, as the available evidence indicates, the presence of rather large amounts of this compound in the urine under certain conditions, to be discussed, must be explained in some other way. For example, in contrast to the results obtained following the ingestion of the free acid, sodium citrate by mouth leads to the appearance of relatively large amounts of citric acid in the urine (Östberg, '31; Schuck, '34 b; Sherman, Mendel and Smith, '36 a). That this difference is due chiefly to the alkali administered is indicated by the fact that equivalent amounts of sodium as sodium bicarbonate produce similar increases in citric acid output (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34; Schuck, '34 b; Sherman, Mendel and Smith, '36 a). On the other hand, the administration of hydrochloric acid and potentially acidic substances, as ammonium and calcium chloride, cause a significant decrease in the excretion of citric acid (Östberg, '31; Kuyper and Mattill, '33; Boothby and

Adams, '34). Similar fluctuations have been observed in total organic acids after acid and alkali administration, as was pointed out in a previous section. Apparently, this is due largely to changes in the amount of citric acid excreted. This constant finding led Östberg ('31) to propose the theory that citric acid is an important 'physiological acid' in the same sense that ammonia is a 'physiological base.' Thus, during periods of alkalosis, citric acid is formed to combine with the excess alkali whereas during acidosis such production is diminished. Some further support to this hypothesis is given by experiments on normal human subjects; the mild alkalosis following hyperpnea and the 'alkaline tide' following meals are accompanied by an increased excretion of citric acid (Kuyper and Mattill, '33).

If citric acid is to function as a physiological acid, it is obvious that either a reserve supply of the substance must be present in the tissues and available for emergencies or that an efficient mechanism must exist for the synthesis of the compound as it is needed. As was pointed out in a preceding paragraph, there is no evidence at the present time of the existence of significant stores of citric acid in animal tissues, at least of the dog and rabbit. On the other hand, the evidence for the endogenous formation of citric acid in the dog appears conclusive. Citric acid is excreted in increased amounts during periods of total starvation (Boothby and Adams, '34). Even more convincing is the finding that large amounts of citric acid were excreted daily for periods exceeding a month by dogs fed a citrate-low basal diet to which alkali was added (Sherman, Mendel and Smith, '36 a). The total amount of citric acid excreted far exceeded that present in the diet and that estimated in the animal's own tissues and body fluids.

As pointed out by Boothby and Adams ('34), there are also indications that citric acid may be involved in physiological processes other than the maintenance of acid-base balance. In support of this statement they cite the fact that in their experiments, increases in the amount of citric acid appearing

in the urine did not necessarily correspond with increases in pH. Even more convincing evidence has been recently reported; the intravenous injection of certain compounds, to be discussed later, caused marked rises in the excretion of citric acid with only slight increases in urinary pH (Orten and Smith, '37). The amount of citric acid excreted after the injection of these compounds was far greater than the quantity eliminated after the injection of equivalent amounts of alkali, whereas the increase in pH was greatest after the alkali administration. These findings suggest that citric acid may be a normal metabolite.

Inasmuch as it appears that citric acid may be formed in the organism as a normal metabolite or in increased amounts in response to alkali administration, questions arise regarding its precursors in the body. Studies of this problem have thus far yielded rather inconsistent results. According to Boothby and Adams ('34), metabolic degradation products of carbohydrates do not necessarily serve as precursors since increased amounts of citric acid are excreted by the dog during starvation at a time when the non-protein respiratory quotient indicates that carbohydrate as such is not being burned. These investigators lean to the view that endogenous citric acid formation is related in some manner to the metabolism of protein since in several instances a rather close parallelism between fluctuations in the amounts of urea and of citric acid excreted in the urine were observed. On the other hand, von Fürth and his collaborators ('34) suggest that metabolic degradation products of carbohydrates in the animal organism may serve as precursors of citric acid, as is thought to be the case in molds and fungi. The fact that in his hand sodium acetate elicited a greater increase in the excretion of citric acid in the pig than did an equivalent amount of sodium bicarbonate was interpreted as evidence in favor of this view.

Other recent work (Sherman, Mendel and Smith, '36 a), however, does not yield convincing evidence that products derived in the organism from either carbohydrate or protein

necessarily serve as precursors of endogenous citric acid. No significant change in the urinary output of citric acid was observed in dogs fed a citrate-low ration when either the amount of carbohydrate or of protein in the diet was increased. However, when alkali was also administered there was an indication that the increased excretion of citrate was augmented by a carbohydrate-rich diet, whereas no such effect followed the feeding of a protein-rich diet. Experiments of the foregoing types are undoubtedly complicated to some extent by such factors as the formation of the same metabolic degradation products from both carbohydrate and protein, acid-base effects of the foods themselves and, in the case of the experiments of von Fürth, by possible varying degrees of intestinal absorption and destruction of the compounds administered.

Recently the problem has been reinvestigated by a method which appears to be free from the foregoing objections (Orten and Smith, '37). A number of simple metabolites and related compounds were injected intravenously into dogs maintained on a constant amount of a citrate-low basal ration, and the effects on the daily urinary excretion of citric acid and the urinary pH were observed. Of all the compounds studied, five were outstanding in producing an increase in citric acid output, namely, the di-sodium salts of dl-malic, maleic, fumaric, succinic and malonic acids. The response from these compounds far exceeded the typical 'alkali effect,' as shown by the fact that equivalent amounts of base as sodium bicarbonate and certain other compounds produced only a relatively small increase in citric acid excretion. Since the above dicarboxylic acids may be derived from degradation products of carbohydrates (Needham, '32), proteins (Needham, '30), or possibly fats (Clutterbuck and Raper, '25), the foregoing experiments suggest that endogenous citric acid may be formed from metabolic derivatives of any of the three 'proximate principles.' The degree of the conversion of such metabolites apparently varies with both the supply of the precursors available and the demand for citric acid to maintain normal acid-base relationships. It may be pointed out

that the above four-carbon atom dicarboxylic acids are probably involved in other physiological processes, as in tissue respiration, and as intermediate compounds in the formation of citric acid by molds and fungi. The frequent inverse relation between the amounts of malic and citric acids in certain fruits and vegetables (Hartmann and Hillig, '34) is of interest in this connection.

The question of variations of citric acid in the blood and urine of human patients in pathological conditions has received some attention. According to Thunberg ('33), the citric acid content of the blood tends to be lower than normal in bronchopneumonia, tetany, pleuritis and erysipelas, whereas normal values are found in pernicious anemia, bronchial asthma, mammary and hepatic carcinoma, diabetes, Basedow's disease, chronic nephritis, acute polyarthritis, cerebral tumor and certain other conditions. Values somewhat higher than normal were frequently observed in diabetes, acute nephritis, polycythemia vera and Basedow's disease. It should be noted, however, that the changes described were not large nor were they always consistent in any one disease. Likewise, no consistent deviations from the normal have been found in the quantity of citric acid excreted in the urine (Östberg, '31; Boothby and Adams, '34), with the exception of diabetes accompanied with acidosis. In the latter condition, low values were usually found. Frequently, however, the excretion of citric acid was diminished in nephritis, pneumonia, severe anemia and purpura, and sometimes also in cardiac decompensation, epilepsy and tetany. It is evident that further studies of this type are needed before final conclusions can be drawn.

OXALIC ACID

Oxalic acid is one of the earliest known organic acids. Although Scheele (see Nordenskiöld, 1892) had worked with the oxalates for some 15 years, it was not until 1785 that he described his success in crystallizing free oxalic acid (Sauerkleesalzsäure) and his identification of it with the acid obtained by oxidizing sugar with nitric acid (Scheele, 1785 b).

The quantitative determination of oxalic acid is based essentially on the fact that the calcium salt is highly insoluble; in this form it is isolated and subsequently estimated either gravimetrically, by titration with permanganate or by the measurement of the carbon dioxide evolved in the course of the permanganate oxidation. However, the complex nature of plant juices and of animal fluids and tissue extracts requires that preliminary steps be taken to isolate, as far as possible, the oxalic acid prior to the final estimation. Arbenz ('17) extracted the finely divided dry material with 15% hydrochloric acid, removed the oxalic acid from this extract with ether and, after repeated precipitation as the calcium salt, finally ignited the precipitate and weighed the calcium oxide. According to Hartmann and Hillig ('34) this is probably as satisfactory as any method thus far suggested for determining oxalic acid in plant material. For its estimation in urine, oxalic acid is precipitated as the calcium salt; from the solution of this calcium oxalate in hydrochloric acid, oxalic acid is extracted with alcohol and/or ether, precipitated again as the calcium salt and determined by titration or by weighing after ignition (see Dakin, '07).

In the determination of oxalate in blood the proteins are first removed by trichloracetic acid or by sodium sulfate. From the filtrate oxalate is precipitated with calcium, and the calcium oxalate oxidized with an excess of permanganate which is then estimated iodometrically (Merz and Maugeri, '31). All such direct titration methods have been criticized by Thomsen ('35) on the ground that it is impossible to adequately remove interfering substances from the oxalate precipitate. He suggested recourse to exhaustive extraction of the preliminary acidified calcium oxalate precipitate in order to isolate as far as possible the uncontaminated oxalic acid. Values obtained with this procedure are much lower than those heretofore given.

Although oxalic acid as a constituent of natural foods is ordinarily associated particularly with rhubarb, available analyses indicate that this substance is very widely distributed, especially in foods of plant origin. Thus it has been

found in potatoes, beans, spinach, beets, tomatoes, cauliflower, onions, mushrooms and celery root, among the vegetables and in currants, raspberries, grapes, pears and prunes, among the fruits. Oxalic acid has also been reported in meat, liver and kidney and in coffee, cocoa and tea. It is striking that the richest sources of oxalic acid in foods are the leaves of some vegetables, but there are such wide differences in concentration that general statements in this connection are unwarranted. Thus, kale, turnip tops, mustard greens and dandelion leaves have little or no oxalic acid, whereas spinach contains some 0.7%, beet greens 0.8% and New Zealand spinach 1.2%. Rhubarb stems, the part of the plant usually eaten, have approximately 0.25% and are thus seen to be by no means the richest common source of this substance. For a more extensive list of values for oxalic acid in food materials, see Arbenz ('17), Ryder ('30) and Kohman ('34). It is of interest that many of the so-called 'acid' fruits like apples, oranges, tomatoes, pineapples and strawberries are comparatively poor in this food acid.

Oxalic acid and the soluble oxalates are poisonous to animals and to most of the higher plants. This has given rise to apprehension regarding the use of certain oxalate-containing foods (see Kohman, '34). Unlike some of the other organic acids occurring in natural foods, notably citric, succinic and malic acids, oxalic acid is apparently not readily oxidized in the animal body in the course of metabolism. Furthermore, owing to the marked insolubility of the calcium salt, there is a tendency to remove calcium ions from body fluids and tissues when soluble oxalates are present. It is the manifold disturbances throughout the organism caused by this removal of calcium, to which the toxic property of oxalic acid is largely due. Inasmuch as ordinary dietary practice insures at least a modicum of calcium in the food, it appears that the oxalic acid of the food which is originally soluble would be precipitated more or less completely and thus not absorbed from the intestine. From the point of view of toxicity, therefore, there would seem to be little danger from the presence of oxalic acid in food.

It has long been recognized that both blood and urine contain oxalic acid. The normal values available in the literature vary considerably owing, doubtless, to the variation in reliability of the methods employed for the determination. Loeper ('33) stated that blood usually contains less than 10 mg. % ; Jürgens and Spehr ('32) found 12.3 mg. % for the fasting dog and 7.4 mg. % for human blood, whereas Thomsen ('35) found only 1.1 mg. % in the blood of rabbits fed with spinach. Suzuki ('34 a) reported the following values in milligrams per cent for the concentration of oxalic acid in the blood of various species: ox, 3.35; pig, 2.95; dog, 1.90; carp, 3.25; sea bass, 3.23; and salmon, 3.27. The level of oxalic acid in the blood appears to be singularly independent of the oxalate content of the food; neither the ingestion of oxalate-rich vegetables nor fasting changes it (Jürgens and Spehr, '32; Athanasiou and Reinwein, '34). The normal values for oxalic acid in human urine range from 9 to 30 mg. per diem. It is of interest that the urine of the dog contains less than does human urine and the same seems to be true for the rabbit, though, as pointed out by Herkel and Koch ('36), it varies with the diet, increasing definitely when oxalate-rich greens are fed.

The constant presence of oxalic acid in blood and urine raises the question of its origin in the body. There seems to be little doubt that soluble oxalates can be absorbed from the intestine; despite the prompt vomiting induced by orally administered oxalic acid, sufficient was absorbed to bring about a sharp rise in the level of oxalate in the blood in experiments of Jürgens and Spehr ('32). Rittmann and Unterrichter ('35) attribute a greater importance to the food as a source of the oxalic acid of the body fluids than has heretofore been done, suggesting that this has not shown in the balance of intake and outgo because of inadequate analyses of the food. Fruits and vegetables in the diet are recognized as important direct sources of oxalic acid due to the oxalate contained therein; succinic, malic and tartaric acids do not give rise to oxalic acid when given by mouth (Jürgens and

Jürgensohn, '34). Although pure proteins do not appear to increase the output of oxalic acid (Salkowski, '00), connective tissue and gelatin have been looked upon as peculiarly effective in inducing an increased excretion of this substance. This observation has been explained by Suzuki ('34 b) by the fact that commercial gelatin contains sufficient oxalic acid to account for the moderate increase in excretion of this substance after the consumption of gelatin. Moreover, neither glycine nor creatine is a precursor of oxalic acid (Dakin, '07; Jürgens and Spehr, '32).

On a diet extremely poor in preformed oxalate or during inanition, this compound continues to be present in the blood and urine. This circumstance has led to the belief that part of the oxalic acid ordinarily appearing in these body fluids is of endogenous origin. The older literature emphasizes the close relation of oxalic acid formation to carbohydrate metabolism and the newer analyses of blood appear to support this conception. Alimentary hyperglycemia, pancreatectomy and clinical diabetes are accompanied by an increase in the level of oxalic acid in the blood as well as in the urine; insulin prevents the oxaluria (DeLucia and Velardi, '29). Loeper ('33) looks upon insulin as the best therapeutic agent for oxalemia. Adrenalin, on the contrary, is said to raise the level of oxalic acid in the blood (Suzuki, '35). The thesis of the interrelation of carbohydrate metabolism and oxalic acid formation is further supported by in vitro studies of simultaneous glycogenolysis and oxalic acid production in tape worms, liver and heart muscle (Loeper, '33).

The influence of individual amino acids on the level of oxalic acid in the blood has been studied by Suzuki ('34 b). Aspartic acid, asparagine and l-amino butyric acid given parenterally to rabbits produced some increase in the level of oxalate in the blood of rabbits, whereas glycine, dl-alanine, l-glutamic acid and d-amino butyric acid had no influence.

A further possible source of oxalic acid is the bacterial activity in the intestine; according to those who consider this an important contribution to the oxalic acid of the body, the

nature of the substrate and the variety of organism present are of significance. A recent study in which Borgström ('36) demonstrated a greater production of oxalic acid in sterile guinea pigs than in normal control animals again raises the question of the importance of intestinal bacteria as a factor in the production of the so-called endogenous oxalic acid. On the other hand, it is not difficult to understand how the activity of these organisms would complicate the interpretation of dietary studies on oxalic acid.

Oxalic acid exerts an influence in nutrition and in physiology in two major directions, both depending largely on its' reaction with calcium. This substance may interfere with the normal utilization of calcium. Götting ('09) cites evidence for changes in the bone of young animals indicating resorption of bone salts as a result of the feeding of oxalic acid. According to Kohman and Sanborn ('35) soluble oxalates withdraw calcium from the body as is shown by the excessive loss in the urine. Again, Fincke and Sherman ('35) observed a marked decrease in calcium utilization in growing rats when spinach provided one-half of the dietary calcium over that when kale was similarly used. These investigators indicated that the oxalate in the spinach was responsible for the interference with the availability of the calcium of the experimental ration. According to Eimer and Bartels ('32) cooking has no effect on the oxalate contained in food. In situations where the calcium of a diet reaches levels admittedly below that dictated by approved nutritional practice, cognizance should be taken of the oxalic acid of the food.

A large part of the renal and ureteral calculi examined contain or consist of calcium oxalate; even the smaller crystals of calcium oxalate may produce tissue injury and hematuria (Braithwaite, '30). The question of oxaluria, its origin and control is therefore of considerable clinical interest and, from the foregoing discussion, appears to be rather closely related to nutrition. Although the output of oxalate in the urine is widely believed to increase in tuberculosis, diabetes and icterus and, latterly, there is some indication that in these

conditions the oxalate level in the blood is elevated, there is little convincing evidence that the output of oxalic acid is influenced one way or the other by renal disease or that urolithiasis is accompanied by marked alteration in the ability of the body to metabolize oxalic acid. The presence of oxalate in the urine depends to a large degree upon the amount of this substance in the diet, the acidity of the gastric juice and the calcium content of the food consumed (Herkel and Koch, '36). Whether or not the highly insoluble calcium oxalate remains in solution in the urine seems to depend upon physical factors, such as the action of non-protein protective colloids, upon adventitious organic foci of precipitation as well as upon the concentration of the salt.

AROMATIC ORGANIC ACIDS

It was pointed out in a foregoing section that there is an increase in the acidity of the urine of human subjects following the ingestion of prunes, plums and cranberries rather than the decrease in acidity expected because of the basic ash yielded by these foods. This apparent discrepancy was first explained (Blatherwick, '14) by the fact that considerable amounts of benzoic acid are present in these foods and that this substance is not oxidized in the organism, as are certain other organic acids, but is excreted as hippuric acid. Subsequent studies suggested (Blatherwick and Long, '23), however, that the amounts of benzoic acid present in the foods were too small to account for the observed excretion of hippuric acid in the urine. Further investigations (Kohman and Sanborn, '31) demonstrated that relatively large amounts, 1.0% on a fresh basis, of another aromatic acid, quinic acid, are present in the prune and cranberry. This compound when fed to man (Quick, '31) was found to be changed in the body to benzoic acid or a closely related substance, and then excreted in the urine as hippuric acid.

Questions arise regarding the fate in the animal body of another aromatic acid, salicylic acid, reported to be present in appreciable amounts in the strawberry, raspberry, blackberry, apricot, crabapple, orange, currant, plum, cherry, apple

and concord grape (see Hartmann and Hillig, '34). Few studies appear to have been made regarding the nutritional and metabolic behavior of this acid. However, it has been reported (Quick, '33) that in contrast to benzoic acid, only a small portion of the salicylic acid ingested by human subjects is conjugated with glycine and excreted as 'salicyluric' acid. Most of the administered compound appears to be excreted as the free acid.

The foregoing studies suffice to indicate that the aromatic organic acids now known to occur in significant amounts in foodstuffs are not oxidized in the organism but are either conjugated with glycine or glycuronic acid and excreted or are eliminated as the free acid. The failure of oxidation of these compounds in the organism apparently accounts for the inability of ingested foods rich in aromatic acids to decrease the acidity of the urine.

OTHER ORGANIC ACIDS

The presence of a number of other organic acids in foods commonly ingested by man has been described (see Hartmann and Hillig, '34). Among these may be mentioned lactic acid in the apple, raspberry, cherry and tamarind; succinic acid in the grape, rhubarb, raspberry, cherry and blackberry; malonic acid in barley, oats, wheat and alfalfa; glyoxylic acid in grape juice; isocitric acid in the blackberry; aconitic acid in barley, maize, rye, wheat and oat plants; and tricarballylic acid in barley and maize plants. Of these, lactic and succinic acids appear to play a significant role in the animal organism. The significance of lactic acid in relation to the metabolism of carbohydrates has been widely investigated and this subject has been adequately reviewed elsewhere. Succinic acid is present in muscle (Needham, '32) and in certain other tissues (Thudicum, 1879; Clutterbuck, '27). This substance may be a degradation product of either carbohydrate (Needham, '32), protein (Needham, '30), or fat (Clutterbuck and Raper, '25; Smedley-Maclean and Pearce, '31). The hypothesis that fumaric acid, formed from succinic acid, plays an

important role as a catalytic link in tissue respiration (Annau et al., '35, '36) indicates the current tendency to attach some physiological significance to the four-carbon atom dicarboxylic acids occurring in tissues. It should be added that as yet, however, there is no unanimity of opinion regarding the alleged role of fumaric acid in tissue respiration.

* * * * * *

The relation of the organic acids present in foods to nutrition and metabolism merits further attention. It seems certain that they are not mere waste products of metabolism in either plants or animals and there is convincing evidence that some of these 'food' acids play fundamental though as yet not well-defined roles in some of the physiological processes of the animal body. It seems well established that several of the organic acids commonly regarded as characteristic constituents of plants are synthesized in the animal body and, in some instances, both the conditions of their formation and their precursors are definitely known. The foregoing account emphasizes the necessity for a consideration of the metabolism of the individual organic acids important in nutrition; only by so doing can a conception of their mode of action and integration in the organism be elucidated.

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THE INFLUENCE OF PARATHYROID HORMONE, UREA, SODIUM CHLORIDE, FAT AND OF INTESTINAL ACTIVITY UPON CALCIUM BALANCE [1]

JOSEPH C. AUB, DOROTHY M. TIBBETTS AND REGINA McLEAN

Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, Boston

The problems of calcium deposit and excretion have been extensively studied in recent years. But the problem of calcium absorption from the intestinal tract has not been adequately investigated or explained. The problem is made difficult by the fact that calcium probably exists in the intestine as a fairly insoluble phosphate, made more insoluble because of the alkaline reaction of the intestinal contents. It is hard to understand, theoretically, why such an insoluble compound should be absorbed at all, though it is obvious that this is accomplished fairly efficiently.

Of the factors which may influence calcium absorption, the need of vitamin D (Bauer, Marble, Claflin, '32 a, '32 b; Gargill, Gilligan and Blumgart, '30) appears to be well established. There is some evidence that the reaction of intestinal contents is also of importance (Abrahamson and Miller, '25; Irving, '26; Bergeim, '26) though this is not definitely established. However, other factors must play a part and a search for them resulted in the following observations.

PARATHYROID HORMONE

In metabolic experiments one must think of intestinal contents as outside of the body. From this point of view the

[1] This is no. XXII in the series of papers entitled "Studies of calcium and phosphorus metabolism."

influence of the parathyroid hormone and of high blood calcium upon fecal calcium can be twofold; for the intestinal tract can act as an area of absorption as well as an area of excretion for body calcium. The great need in the body for calcium in hyperparathyroidism might well stimulate calcium absorption, while the high blood calcium level in this disease might not only interfere with calcium absorption but might also accentuate body calcium elimination into the feces. Let us consider these two effects.

1. Excretion into the intestinal tract might be accentuated. This can be shown only when a low calcium intake has been used—so that the fecal excretion of calcium is distinctly higher than the ingestion. Much has now been published to indicate that the injection of parathyroid extract does not usually increase fecal calcium excretion (Greenwald and Gross, '26; Brehme and György, '27; Stewart and Percival, '27). When parathyroid extract was administered to our previous cases on a low calcium intake, the fecal calcium excretion was shown to be within normal limits (Albright, Bauer, Ropes and Aub, '29). In a recent observation on a patient convalescent from lead intoxication (E.M., tables 1 and 2), with a constant low calcium diet, the fecal calcium in four control periods averaged 505 mg., while during nine periods of parathyroid extract administration, when the blood calcium was elevated to between 12 and 15 mg.%, the fecal calcium averaged 515 mg. per period. In two cases of parathyroid adenoma reported here (table 1), one, G.M., showed a normal fecal calcium excretion, while on a low calcium intake; the other, A.R. (as also Charles M., reported by Bauer, Albright and Aub, '30), was found to have a very low output of calcium in the feces on a similar diet. This lack of influence, however, has not been universally found, for several cases of parathyroid adenoma have been reported in the literature as abnormal in their fecal excretion. Hunter ('29) found an excessive amount of calcium in the stools of one patient, and of the three cases studied by Bulger, Dixon, Barr and Schregardus ('30), two showed an abnormally high output of calcium in the feces.

TABLE 1

Effect of parathyroid hormone on excretion and absorption of calcium
(output and intake in 3-day periods)

SUBJECT	PERIOD	DATE	DIET AND MEDICATION PER PERIOD	CALCIUM			SERUM VALUES Ca
				Excretion		Intake	
				Urine	Feces		
				gm.	*gm.*	*gm.*	*mg. %*
E.M. Normal control	I	Sept. 1933 14–16	Low calcium	0.03	0.55	0.30	9.8
	II	17–19	Low calcium	0.08	0.43	0.30	10.0
	III	20–22	Same + 1500 units	0.08	0.52	0.30	11.9
	IV	23–25	parathyroid extract	0.84	0.48	0.30	16.4
	V	26–28	Same + 750 units	1.34	0.61	0.30	17.1
	VI	29– Oct. 1	parathyroid extract	1.13	0.44	0.30	15.3
G.M. Parathyroid adenoma	I	May 1932 18–20	Low calcium	1.95	0.48	0.29	19.4
	II	21–23	Low calcium	2.15	0.50	0.29	...
	III	24–26	High calcium	2.04	4.03	4.64	...
	IV	27–29	High calcium	2.27	3.15	4.63	18.6
	V	30– June 1	High calcium	2.14	3.06	4.62	...
	Parathyroidectomy—June 4, 1932						
	VI	June 12–14	High calcium	0.04	3.59	4.76	8.7
	VII	15–17	High calcium	0.02	3.20	4.76	7.1
	VIII	18–20	High calcium	0.02	3.12	4.76	7.5
	IX	21–23	High calcium	0.02	2.32	4.76	7.5
	Second admission—September 8, 1932						
	XI	Sept. 14–16	High calcium	0.02	1.60	4.76	10.4
	XII	17–19	High calcium	0.02	1.67	4.76	10.4
	XIII	20–22	Low calcium	0.02	0.10	0.29	...
	XIV	23–25	Low calcium	0.01	0.11	0.29	...
	XV	26–28	Low calcium	0.01	0.09	0.29	9.7
A.R. Parathyroid adenoma	II	April 1932 5– 7	High calcium	0.99	2.38	5.84	10.7
	III	8–10	High calcium	1.00	2.10	5.84	...
	IV	11–13	High calcium	0.92	1.90	5.84	11.2
	Second admission—October 17, 1932						
	VI	Oct. 1932 21–23	Low calcium	0.62	0.12	0.29	13.6
	VII	24–26	Low calcium	0.75	0.13	0.29	...

TABLE 1—*Continued*

SUBJECT	PERIOD	DATE	DIET AND MEDICATION PER PERIOD	CALCIUM			SERUM VALUES Ca
				Excretion		Intake	
				Urine	Feces		
A.R.				*gm.*	*gm.*	*gm.*	*m.%*
Para-thyroid adenoma	IX	30–Nov. 1	High calcium	1.93	0.66	5.84	...
	X	2–4	High calcium	2.10	1.06	5.84	13.0
	XI	5–7	High calcium	2.08	1.08	5.84	13.6
Parathyroidectomy—December 2, 1932							
Third admission—January 18, 1933							
		1933 Jan.					
	XIII	26–28	Low calcium	0.02	0.30	0.29	9.8
	XIV	29–31	Low calcium	0.02	0.19	0.29	9.9
		Feb.					
	XVI	5– 7	High calcium	0.13	2.08	5.84	...
	XVII	8–10	High calcium	0.19	2.92	5.84	9.8
F.G. Para-thyroid adenoma		1935 Jan.					
	I	20–22	Moderate calcium	1.36	1.13	2.20	15.5
	II	23–25	Moderate calcium	1.45	0.99	2.20	14.2
Parathyroidectomy—February 8, 1935							
Second admission—September 14, 1936							
		1936 Sept.					
	VII	18–20	Moderate calcium	0.14	1.70	2.20	10.0
	VIII	21–23	Moderate calcium	0.19	2.02	2.20	9.6

2. Absorption of calcium from the intestinal tract may be influenced. Since the parathyroid has been repeatedly demonstrated to have no influence on body calcium elimination in the feces, any change in fecal excretion produced by parathyroid over-activity during high calcium feeding would obviously be dependent upon variations in calcium absorption. In order to solve this problem, three classical cases of hyperparathyroidism were studied before and after the removal of the parathyroid tumor. The fecal elimination during identical regimes gives the necessary comparison—the periods of low calcium intake establishing as nearly as practicable the intestinal excretion, while the high calcium periods then give an index of calcium absorption.

Methods. Our usual methods previously described (Bauer and Aub, '27), have been used both as regards the diet and the metabolic ward procedure. All the diets were essentially neutral in reaction with constant water and sodium chloride content. The patients here discussed were perfect metabolic subjects, so that no errors occurred during the observations, and they ate their total diet daily so that no corrections for uneaten food had to be made. The case histories and additional metabolic data may be found in papers by Tibbetts and Aub ('37 a, b).

Results. The results of these observations indicate that the parathyroid secretion exerts a relatively minor influence upon intestinal calcium absorption as well as fecal calcium excretion.

In the patient G.M. (fully reported by Tibbetts and Aub, '37 a, b), the first postoperative observation was started 8 days after the operation and 3 days after returning to the full diet. On the high calcium diet, the fecal calcium excretion in the first postoperative periods showed gradually diminishing values, though the average of all four periods fell only about 10% of the preoperative figures in spite of the dramatic drop in blood calcium level. The fecal phosphorus fell an analogous amount in spite of no change in inorganic phosphorus blood level (Tibbetts and Aub, '37 a, b).

Three months later, when the patient was growing rapidly, a new mechanism had obviously come into play, and both urinary and fecal calcium were being avidly retained in spite of higher blood levels above the so-called kidney threshold (Albright and Ellsworth, '29; Aub, Albright, Bauer and Rossmeisl, '32). This is an experience encountered twice before in growing boys who had essentially no disease (Albright, Bauer, Ropes and Aub, '29; Farquharson, Salter, Tibbetts and Aub, '31). The need for calcium could hardly be greater during growth than during the active decalcification of hyperparathyroidism, yet this new mechanism which improves absorption and prevents loss of calcium in the feces developed slowly and was not manifest during the periods of over-activity

of the parathyroids. That it cannot be ascribed to vitamin D seems clear as this patient received 5 drops of viosterol per day for 2 years previous to operation, and only 10 drops daily between the first and second postoperative studies. The two growing boys, previously reported, received no added vitamin D.

With the patient A.R., this investigation is not so clearly defined, as the first observation was made after a series of x-ray therapy to the pelvis. This produced an obvious though temporary fall in serum calcium levels and the calcium excretion at this time is shown in the results of her first observation. These data show that though the urinary excretion is high, the fecal excretion is only a little lower than that found after operation.

However, in the second admission, when the serum calcium had approached the pre-x-ray level (14.4 mg.%), a different situation occurred for the urinary excretion had risen markedly at the expense of only the fecal calcium while the balance remained the same in the two observations.

After the removal of the parathyroid adenoma, there is poorer absorption and a greater excretion of calcium from the intestine, while there is the usual dramatic fall in urinary excretion. This is true both in the high calcium and in the low calcium periods.

The observations on the patient F.G. were exactly analogous. The second observation was made 19 months after the parathyroidectomy was performed. During this period the patient was on a very high calcium intake, and, though there were still some bone cysts present the x-rays demonstrated much more normal bones. On exactly the same diet in both observations, the fecal excretion of calcium and phosphorus is higher in the postoperative periods. Therefore, these two observations indicate that the absorption of calcium from the intestine was greater during the hyperparathyroid state.

In the clear-cut studies of three patients reported here there is better intestinal absorption of calcium during the hyperparathyroid state in the two adult cases and less absorption

in the adolescent boy. It, therefore, appears clear that the effects of removal of a simple adenomatous parathyroid tumor causes inconstant effects upon fecal absorption and excretion. If the parathyroids were directly involved in intestinal absorption of calcium, one would expect the results to be consistent in all three of these classical examples of hyperparathyroidism. The inconstancy of the findings would appear to justify the conclusion that the parathyroid secretion has notably little to do with calcium absorption, but the dramatic changes which occurred in the adolescent boy make it clear that there are other, unknown but very potent factors which may greatly influence fecal calcium absorption.

Other factors which might influence calcium absorption which we have studied include substances which might affect the solubility of molecules in body fluids or substances which influence the bulk and movements of the intestinal contents.

UREA

Of substances which might affect solubility in body fluids, urea seemed very likely to be of value. This decision was reached because Cohn ('32, '35, '36) has shown that, along with amino acids and proteins, urea has a great solvent action on inorganic ions as well as on the dipolar ions of organic compounds. It is also a normal and widely distributed constituent of the body. There is also the evidence produced by Gamble, McKhann, Butler and Tuthill ('34) that the concentration of some urinary constituents can be increased by the addition of urea to the diet. They, however, did not study urinary calcium. It, therefore, seemed possible that increased concentration of urea in body fluids might increase the solubility of calcium and thereby affect the intestinal absorption, blood level and kidney excretion.

Methods. The metabolic methods of study were similar to those in the preceding report except as follows: Urea was given daily, usually as three doses of 10 to 25 gm. each, by mouth. It was dissolved in water, and because of its great thirst-producing influence, water intake during its use was

TABLE 2

Effect of urea ingestion (output and intake in 3-day periods)

SUBJECT	PERIOD	MEDICATION PER PERIOD	VOLUME OF URINE	CALCIUM			PHOSPHORUS			UREA NITROGEN		SERUM		WHOLE BLOOD NPN
				Excretion		Intake	Excretion		Intake	Excretion in urine	Added to diet	Ca	P	
				Urine	Feces		Urine	Feces						
			cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc.	mg./100 cc.
A.D. (normal control)	XV	Control	5420	0.45	0.50	0.32	2.76	0.79	3.17	....	....	11.0	4.2	..
	XVI	Control	6480	0.47	0.31	0.32	2.73	0.54	3.17	....	....	...	...	..
	XVII	Control	5990	0.55	0.35	0.32	2.80	0.66	3.12	34.3	....	...	...	28
	XVIII	90 gm urea	6480	0.62	0.44	0.32	2.48	0.86	3.17	72.3	42.0	...	...	..
	XIX	150 gm. urea	5210	0.67	0.33	0.32	2.59	0.64	3.16	99.3	70.0	...	...	..
	XX	150 gm. urea	7250	0.80	0.35	0.32	2.68	0.73	3.17	102.2	70.0	10.8	4.0	36
	XXI	Control	6520	0.68	0.32	0.32	2.51	0.65	3.17	35.8	....	...	...	..
	XXII	Control	5960	0.66	0.38	0.32	2.51	0.85	3.17	....	....	11.0	4.4	30
C.W. (normal control)	XV	Control	6530	0.33	0.67	0.32	2.65	0.85	3.17	....	....	10.2	4.5	..
	XVI	Control	6900	0.40	0.51	0.32	2.77	0.67	3.17	....	....	...	...	..
	XVII	Control	7130	0.36	0.60	0.32	2.63	0.75	3.16	34.2	....	10.8	4.2	..
	XVIII	90 gm. urea	6980	0.43	0.76	0.32	2.78	0.07	3.17	76.3	42.0	...	...	..
	XIX	150 gm. urea	6340	0.51	0.58	0.32	2.50	0.81	3.17	99.4	70.0	...	...	..
	XX	150 gm. urea	7330	0.57	0.54	0.32	2.36	0.71	3.17	95.8	70.0	10.3	3.8	37
	XXI	Control	6780[1]	0.49	0.61	0.32	2.81	0.83	3.17	40.4	....	...	...	..
	XXII	Control	6890	0.46	0.49	0.32	2.49	0.61	3.17	....	....	10.5	4.4	31
E.M. (normal control)	VIII	750 units parathyroid extract + 225 gm. urea	8910	0.67	0.63	0.31	1.42	0.89	2.23	92.4	105.0	13.9	3.0	75
Huntington Hospital No. 33-1159	XI	750 units parathyroid extract + 225 gm. urea	8480	0.47	0.55	0.31	1.51	0.97	2.23	116.0	105.0	12.9	3.1	76
	X	750 units parathyroid extract	7380	0.43	0.41	0.31	1.68	0.97	2.23	39.8	....	12.7	...	38
	XI	750 units parathyroid extract	7190	...	0.41	0.31	2.06	0.80	2.23	20.3	....	12.0	3.5	..

Patient	Period	Treatment												
M.T. (hyperparathyroidism) Huntington Hospital no. 33-619	XII	Control	7660	0.23	0.56	0.31	0.57	1.08	2.23	....	....	10.8	..	..
	XIII	Control	6790	0.09	0.48	0.31	1.03	1.11	2.23	....	....	9.8	3.5	..
	I	Control	3580	0.42	1.20	1.56	0.93	0.52	1.55	14.1	....	13.7	2.4	..
	II	Control	2810	0.43	0.86	1.56	0.77	0.43	1.55	10.0	....	..	..	..
	III	225 gm. urea	7000	0.43	0.83	1.56	0.82	0.45	1.55	95.0	105.0	13.9	1.6	57
	IV	140 gm. urea	5690	0.53	1.20	1.56	1.11	0.52	1.55	82.0	65.0	..	..	63
	V	135 gm. urea	4780	0.56	0.85	1.56	1.01	0.56	1.55	69.6	63.0	16.0	..	68
	VI	Control	1880	0.33	0.88	1.16	0.96	0.33	1.03	29.9	....	13.3	1.5	49
	VII	Control	4110	0.42	0.81	1.53	0.74	0.52	1.55	10.2	....	13.0	1.4	33
R.B. Lahey Clinic no. 33022 (hyperparathyroidism)	I	Control	2450	0.56	..	0.18	..	..	..	....	....	14.0	..	..
	II	Control	3110	0.72	..	0.20	..	..	..	11.7	....	14.7	2.2	..
	III	208 gm. urea	7510	0.85	..	0.20	..	..	..	90.4	98.0	..	..	..
	IV	208 gm. urea	6190	0.85	..	0.20	..	..	..	95.0	97.0	15.8	1.8	80
A.R. Huntington Hospital no. 32-291 (after parathyroidectomy)	XVI	Control	3820	0.13	2.08	5.44	3.44	0.91	6.44	....	....	..	..	..
	XVII	Control	4280	0.19	2.92	5.44	3.50	1.20	6.44	45.8	....	10.2	4.4	39
	XVIII	225 gm. urea	6920	0.21	2.59	5.44	3.69	1.17	6.44	156.9	105.0	..	..	..
	XIX	225 gm. urea	6740	0.21	2.24	5.44	3.64	0.89	6.44	146.5	105.0	9.9	4.2	42
E.D. Huntington Hospital no. 33-370 (myxedema)	I	Control	5380	0.37	0.22	0.31	1.42	0.50	1.86	20.6	....	10.0	3.1	..
	II	Control	5140	0.37	0.21	0.31	1.49	0.48	1.86	21.1	....	10.0	..	30
	III	225 gm. urea	6120	0.41	0.31	0.31	1.59	0.86	1.86	84.5	105.0	10.0	..	..
	IV	225 gm. urea	5860	0.43	0.23	0.31	1.28	0.59	1.86	118.3	105.0	..	..	..
	V	225 gm. urea	6200	0.44	0.32	0.31	1.22	0.79	1.86	114.4	105.0	10.2	3.1	..
M.H. Huntington Hospital no.33-146 (carcinoma of thyroid)	I	Control	3780	0.48	1.18	1.20	1.25	0.90	2.44	15.1	....	9.7	4.6	32
	II	Control	3970	0.49	1.24	1.20	1.23	1.01	2.44	15.0	....	..	..	..
	III	150 gm. urea	4800	0.58	1.22	1.20	1.31	1.04	2.44	71.5	70.0	..	..	..
	IV	225 gm. urea	5890	0.59	1.10	1.20	1.30	0.77	2.44	119.9	105.0	..	..	..
	V	225 gm. urea	5800	0.66	1.24	1.20	1.54	0.74	2.44	117.0	105.0	10.0	4.5	50

¹ Two-day specimen calculated for 3-day period.

TABLE 3

Effect of urea ingestion on serum calcium

SUBJECT	DATE	TIME	SERUM		WHOLE BLOOD NPN	REMARKS
			Ca	P		
			mg./ 100 cc.	*mg./ 100 cc.*	*mg./ 100 cc.*	
T.P. P.B.B.H. no. 43292 med. hyper- thyroidism	1933 July 17	Fasting	9.8	3.5	28	Control
	July 20	Fasting	10.8		33	75 gm. urea on July 18 and 19
	July 24	Fasting	10.5	3.5	33	75 gm. urea daily July 20th to 24th
E.F. P.B.B.H. no. 44094 surg. hyper- thyroidism	July 17	Fasting	10.2		34	Control
	July 20	Fasting	11.4		46	75 gm. urea on July 18 and 19
	July 24	Fasting	11.0		41	75 gm. urea daily July 20th to 24th
H.S. M.G.H. no.330547 Hyper- thyroidism	July 17	Not fasting	10.0	3.3		Control
	July 21	Fasting	10.0		34	Control
	July 24	Fasting	10.5		33	75 gm. urea daily July 21st to 24th
	July 27	Fasting	10.6		37	75 gm. urea on July 25th and 26th
		1 hour after urea	10.9		75	
H.M. M.G.H. no. 333961 Hyper- thyroidism	Dec. 15		11.2		35	Control
	Dec. 16		10.8	4.9	39	Control
	Dec. 17		10.8		38	75 gm. urea daily Dec. 16th to 21st
	Dec. 20		11.2			
	Dec. 22		11.5		39	90 gm. urea daily Dec. 22nd to 26th
	Dec. 23	Fasting	11.3	4.8	38	
		1 hour after 30 gm. urea	11.2	5.0	60	
	Dec. 26		11.4			
	Dec. 27		11.3			Control
	Jan. 3		11.0	4.8		Control

TABLE 3—*Continued*

SUBJECT	DATE	TIME	SERUM		WHOLE BLOOD NPN	REMARKS
			Ca	P		
			mg./ 100 cc.	*mg./ 100 cc.*	*mg./ 100 cc.*	
	July 17	After breakfast	11.1	2.3		Control
	July 19	After breakfast	11.6	2.0	33	Control
	July 20	Fasting	11.5			25 gm. urea night before
F.C. B.C.H. no. 713282	July 24	Fasting	11.6	2.0	24	Small doses of urea July 20th to 24th
Hyperpara- thyroidism	July 28	Fasting	12.4	2.3	27[1]	75 gm. urea daily July 25th to 31st
	July 28	45 minutes after urea	12.7		63[1]	
	July 31	Fasting	12.7		24	
.		45 minutes after urea	12.9		49	
D.B.	1934					Control
M.G.H.	Feb. 6		5.7	7.4		30 gm. urea Feb. 6
no. 334687	Feb. 7					45 gm. urea Feb. 7
idiopathic	Feb. 8					60 gm. urea Feb. 8
hypopara- thyroidism	Feb. 9	2 hours after urea	6.8	7.7		20 gm. urea Feb. 9

[1] Serum NPN.

not kept constant but was left to the desire of the patient, except in the case of two normal controls.

Results. The observations need but little discussion, for the tables speak for themselves. The data give some evidence of an effect of urea feeding upon blood calcium levels. This is not evident in the normal controls, even when the blood calcium level has already been elevated by the injection of parathyroid extract (E.M., table 2). But in cases with hyper-parathyroid adenomas and in hyperthyroidism (which has an abnormally high calcium excretion (Aub, Bauer, Heath and Ropes, '29)), the addition of urea gives an indication in all

cases of a slight increase in blood calcium concentration (tables 2 and 3).[2]

It is clear that urea taken in large quantities by mouth does not markedly increase calcium absorption from the intestine. This is true in the observations with a low as well as with a high calcium content in the diet, and is also true in conditions in which there is a need for increased calcium absorption, such as in hyperparathyroidism (patients E.M. and M.T.), or in recovery after parathyroidectomy (A.R.).

In relation to urinary excretion the evidence points to a definite elevation of calcium excretion during the ingestion of urea, though this is not of great magnitude. This elevation is probably not surprising when one thinks of the huge substrate of calcium available for solution and for continued excretion, if urea increased the solubility in blood. The increase was found even in the two normal controls (table 2) in whom, because of a constant fluid intake, no diuresis occurred. In the other subjects, whose fluid intake was not restricted, there occurred a large increase in urinary volume because of the great thirst produced by urea ingestion. Associated with this diuresis the balance experiments show an elevation of urinary calcium in all observations, of the same magnitude as in the two controls who had no increased urinary output. Taken from the point of view of urinary concentration, the observations indicate the comparative independence of calcium excretion in relationship to urinary volume.

This independence in excretion of calcium is also obvious in M.T. (see table 2 and appended case history). Her bones were so extraordinarily thin that it was only with very great difficulty that x-ray pictures could be obtained. They showed multiple cysts and multiple spontaneous fractures due to prolonged hyperparathyroidism. Because of her severe nausea

it was considered unwise to give her the rigid routine low calcium diet. On her moderate calcium diet (520 mg. Ca per day), it can be seen that her calcium output is not abnormally high, for a normal control who received 700 mg. of calcium per day excreted 390 mg. per period in urine and 980 mg. in feces (Farquharson, Salter, Tibbetts and Aub, '31). Here, then, is a patient with proven hyperparathyroidism, who had, as a result, a diminished calcium reserve of truly remarkable degree. Her enormous need for calcium was not accompanied by great retention of calcium from the bowel, which reacted as in a normal individual. While still capable of maintaining a normal fecal excretion and a markedly elevated blood calcium level, there was still some mechanism present which prevented the usual lavish loss of urinary calcium. This independence of blood elevation and urinary excretion has been previously reported in patients with severe nephritis by Albright, Baird, Cope and Bloomberg ('34). But this patient did not appear to have a nephritis adequate to account for this change in excretion. In spite of this protective mechanism which prevented the great loss of urinary calcium usually associated with hyperparathyroidism, the addition of urea to the diet definitely increased both blood calcium and urinary calcium excretion.

It therefore seems clear that the additional ingestion of large amounts of urea has practically no effect on calcium absorption from the bowel. However, it increased urinary calcium excretion in all observations, including a case extraordinarily depleted of calcium. The effect of urea on the blood was negligible in normal controls, but caused an elevation in hyperthyroid and hyperparathyroid patients.

INTESTINAL ACTIVITY

In the previous sections of this paper were reported the influence of the parathyroid hormone on calcium absorption and the effects of urea ingestion on absorption and excretion of calcium. Other factors which might well influence calcium exchange exist in the bulk and rapidity of fecal

elimination, as well as the interrelation with other inorganic salts. The problem can be approached in two ways: either in regard to absorption, or in regard to intestinal excretion. In these studies, the latter approach was chosen and, therefore, the two subjects (normal medical students) were given a diet adequate to their needs except in regard to calcium.

The purposes of these observations were to determine the effects on calcium excretion of:

1) Variations in bulk and fluid of the feces. 2) The effect of a high fat diet, which, by the formation of calcium soaps, might increase fecal calcium. 3) The effect of urea—already discussed. 4) The influence of large amounts of another physiological salt—sodium chloride.

The two medical students were perfect subjects, and the results are given in full in order to be available as reference for future investigations in this field. Blood calcium and phosphorus values did not show significant changes and are not recorded.

It is to be expected that diarrhea produced by cascara would increase the total base excretion in the feces, and this is accompanied by a similar fall in urinary excretion. But this increased fecal total base excretion is probably largely sodium, for voluntary constipation (periods I–III) and diarrhea (periods IV–VII), and the addition of agar (periods XXIII–XXVI) to the diet caused no significant variation in either fecal or urinary calcium in spite of the great shifts in the wet weight of the feces. Just as calcium excretion appeared independent of urinary diuresis, so here it is independent of fecal volume.

The addition of fat to the diet (approximately 200 gm. per day in the form of butter fat and olive oil) also caused no increased calcium excretion in the feces (periods XII–XIV). The high fecal calcium found in steatorrhea, therefore, represents a wide divergence from the normal inasmuch as we were unable to reproduce it in our normal controls. This period of high fat feeding, however, caused a slight elevation

TABLE 4

A.D. (normal control) (output and intake in 3-day periods)

LOW CALCIUM DIET THROUGHOUT TREATMENT (PER PERIOD)	PERIOD[1]	TOTAL VOLUME OF DRINK (cc.)	WEIGHT OF WET STOOLS (gm.)	CALCULATED EXCESS BASE IN DIET[2] (cc. N/10)	TITRATABLE ACIDITY + AMMONIA IN URINE (cc. N/10)	NITROGEN Excretion urine (gm.)	NITROGEN Intake (gm.)	CALCIUM Excretion urine (gm.)	CALCIUM Excretion feces (gm.)	CALCIUM Intake[3] (gm.)	PHOSPHORUS Excretion urine (gm.)	PHOSPHORUS Excretion feces (gm.)	PHOSPHORUS Intake[3] (gm.)	TOTAL BASE Excretion urine (cc. N/10)	TOTAL BASE Excretion feces (cc. N/10)	TOTAL BASE Intake[2] (cc. N/10)	CHLORIDE Excretion urine (cc. N/10)	CHLORIDE Intake[3] (cc. N/10)	SERUM Ca (mg.)	SERUM P (mg.)
Voluntary constipation	I	5870	274	−195	1606	40.8	43.2	0.47	0.45	0.31	2.23	0.66	3.30	4680	910	5199	2340	1870		
	II	5190	153	−195	1426	39.8	43.2	0.47	0.33	0.31	2.23	0.54	3.30	4320	685	5199	2270	1870	10.4	4.0
	III	6230	237	−195	1405	39.7	43.2	0.47	0.46	0.31	2.50	0.85	3.30	4250	1620	5199	2580	1870		
20 cc. cascara	IV	5500	681	−195	1526	38.2	43.2	0.46	0.52	0.31	2.27	0.95	3.30	4100	990	5445	2360	1870		
33 cc. cascara	V	4570	680	−195	1539	36.0	43.2	0.45	0.43	0.31	1.94	0.72	3.30	3330	1260	5605	1755	1870		
51 cc. cascara	VI	5470	884	−195	1643	35.5	43.2	0.53	0.44	0.31	2.34	0.72	3.30	4100	1730	5949	2690	1870		
71 cc. cascara	VII	5710	1316	−195	1783	36.7	43.2	0.57	0.51	0.31	2.34	0.80	3.30	3520	2740	6069	2160	1870		
Control	VIII	5710	344	−195	1755	38.8	43.2	0.47	0.34	0.31	2.44	0.54	3.30	3480	870	5199	2020	1870	10.5	4.0
	IX	5990	398	−195	1347	40.2	43.2	0.49	0.44	0.31	2.24	0.69	3.30	4240	1090	5199	2300	1870		
	X	5500	496	−139	1889	40.6	37.2	0.43	0.37	0.27	2.70	0.66	2.86	3080	990	4487	1100	1640	10.5	
	XI	6570	426	−195	1410	39.4	43.2	0.52	0.44	0.31	2.34	0.75	3.30	4050	1250	5199	1815	1870		
100 gm. added fat	XII	4940	328	−36	1415	40.4	43.3	0.40	0.43	0.30	2.30	0.60	3.20	3100	980	5146	1105	1870	11.0	4.2
100 gm. added fat	XIII	4770	544	−72	1691	37.5	43.0	0.39	0.74	0.31	2.02	0.93	3.11	2790	1580	5112	990	1870		
750 gm. added fat	XIV	5935	378	−72	1749	35.8	43.2	0.47	0.50	0.31	2.36	0.58	3.11	3420	1070	5112	1520	1870		
5 gm. Merck's agar	XXIII	6180	450	−143	1185	34.6	35.1	0.57	0.46	0.26	2.36	0.98	3.34	3880	1090	4205	1560	1380	11.0	4.4
5 gm. Merck's agar	XXIV	5260	262	−195	1186	33.2	35.5	0.52	0.30	0.26	2.10	0.49	2.59	3160	530	4205	1190	1380		
5 gm. Bacto-agar	XXV	4980	446	−243	1222	37.8	43.2	0.66	0.11	0.32	2.15	0.50	2.65	3880	730	5270	1630	1870		
5 gm. Bacto-agar	XXVI	6790	382	−243	1412	40.5	43.2	0.75	0.13	0.32	2.25	0.64	2.89	3800	690	5270	1890	1870		
Control	XXVII	6620	302	−243	1136	41.6	43.2	0.64	0.31	0.32	2.25	0.64	2.89	4500	670	5142	1435	1870	11.8	4.2
+ 30 gm. NaCl	XXVIII	4700	242	−243	1259	40.2	43.2	0.70	0.35	0.32	2.70	0.65	3.35	11500	760	10272	5055	6990		
+ 40 gm. NaCl	XXIX	4930	282	−243	1367	37.8	43.2	0.66	0.35	0.32	2.34	0.64	2.98	9450	770	11982	8995	8690	11.1	4.4
+ 45 gm. NaCl	XXX	6800	390	−161	1297	37.2	38.8	0.69	0.31	0.28	2.34	0.51	2.85	10560	690	12257	9135	9350		
Control	XXXI	5190	246	−243	1654	38.8	43.2	0.51	0.29	0.32	2.18	0.42	2.60	7700	550	5142	1645	1860		

[1] Periods XV to XXII in table 2.
[2] Calculated from Sherman's tables (27).
[3] Calculated from analyses of diets and individual foodstuffs made in this laboratory.

TABLE 5

C.W. (normal control) (output and intake in 3-day periods)

LOW CALCIUM DIET THROUGHOUT TREATMENT (per period)	PERIOD[1]	TOTAL VOLUME OF URINE (cc.)	WEIGHT OF WET STOOLS (gm.)	CALCULATED EXCESS BASE IN DIET[2] (cc. N/10)	TITRATABLE ACIDITY + AMMONIA IN URINE (cc. N/10)	NITROGEN Excretion urine (gm.)	NITROGEN Intake[3] (gm.)	CALCIUM Urine (gm.)	CALCIUM Feces (gm.)	CALCIUM Intake[3] (gm.)	PHOSPHORUS Urine (gm.)	PHOSPHORUS Feces (gm.)	PHOSPHORUS Intake[3] (gm.)	TOTAL BASE Urine (cc. N/10)	TOTAL BASE Feces (cc. N/10)	TOTAL BASE Intake[3] (cc. N/10)	CHLORIDE Excretion urine (cc. N/10)	CHLORIDE Intake[3] (cc. N/10)	SERUM Ca (mg.)	SERUM P (mg.)
Voluntary constipation	I	5085	299	−162	1794	40.6	43.2	0.21	0.60	0.32	2.02	0.84	3.31	3340	950	5202	1780	1870		
	II	5760	286	−162	1646	42.9	43.2	0.26	0.64	0.32	2.23	1.05	3.31	4500	1140	5202	1950	1870	10.4	4.3
	III	6930	259	−162	1635	44.5	43.2	0.33	0.54	0.32	2.28	0.86	3.31	5300	1050	5202	2160	1870		
11 cc. cascara	IV	6530	879	−162	1765	42.9	43.2	0.34	0.73	0.32	2.40	1.18	3.31	4100	2000	5337	1880	1870		
24 cc. cascara	V	5800	1065	−162	2228	41.7	43.2	0.30	0.56	0.32	2.24	0.86	3.31	3100	1930	5497	2250	1870		
21 cc. cascara	VI	5360	1106	−162	2348	42.2	43.2	0.35	0.62	0.32	2.30	1.10	3.31	3420	2180	5460	1520	1870	10.2	4.4
10 cc. cascara	VII	6660	975	−162	2385	43.8	43.2	0.44	0.58	0.32	2.50	0.80	3.31	3380	1840	5325	2380	1870		
Control	VIII	6210	458	−162	2012	42.4	43.2	0.44	0.65	0.32	2.40	0.90	3.31	3400	1320	5202	2060	1870		
	IX	6110	431	−162	1732	44.1	43.2	0.38	0.52	0.32	2.58	0.80	3.31	4000	1160	5202	1950	1870	10.7	
	X	6420	506	−127	2238	44.7	44.0	0.35	0.69	0.32	2.70	1.14	3.35	2950	1520	5307	1450	1950		
	XI	6920	394	−162	1969	40.7	43.2	0.42	0.44	0.32	2.34	0.71	3.31	3560	1040	5202	1910	1870		
600 gm. added fat	XII	6370	782	−97	1735	39.9	43.3	0.38	0.71	0.31	1.88	0.98	3.25	2630	1680	5273	1550	1870	10.2	4.5
600 gm. added fat	XIII	6550	534	−51	2007	42.8	42.8	0.38	0.62	0.32	2.26	0.73	3.12	2850	1290	5115	1200	1870		
650 gm. added fat	XIV	6420	434	−51	1863	40.5	43.2	0.32	0.56	0.32	2.36	0.65	3.12	3420	1250	5115	1700	1870		
15 gm. Merck's agar	XXIII	6680	442	−192	1515	36.7	35.7	0.41	0.58	0.27	2.27	0.76	2.60	3340	1120	4205	1580	1380	10.5	4.4
15 gm. Merck's agar	XXIV	6390	440	−227	1536	36.0	36.4	0.38	0.08	0.29	2.00	0.70	2.65	2500	900	4258	1210	1430		
45 cc. Bacto-agar	XXV	5780	556	−222	1776	41.0	43.2	0.48	0.44	0.32	2.00	0.91	3.17	3060	1430	5303	1310	1870	10.2	
45 cc. Bacto-agar	XXVI	7640	514	−222	1719	42.8	43.2	0.51	0.63	0.32	2.08	0.77	3.17	3040	1350	5303	1650	1870		
Control	XXVII	7640	310	−222	1521	41.9	43.2	0.48	0.65	0.32	2.22	0.84	3.17	3680	1120	5145	1520	1870	10.4	4.0
30 gm. NaCl	XXVIII	6110	388	−222	1428	42.7	43.2	0.52	0.55	0.32	2.35	0.69	3.17	7340	940	10275	5360	6990		
40 gm. NaCl	XXIX	6070	400	−224	1526	40.9	43.2	0.52	0.57	0.32	2.30	0.82	3.09	9340	1060	11939	8200	8690		
45 gm. NaCl	XXX	7520	242	−222	1527	40.5	43.2	0.53	0.46	0.32	2.27	0.61	3.17	10080	870	12840	9460	9490	10.5	4.4
Control	XXXI	6460	370	−222	2067	45.2	43.2	0.31	0.49	0.32	2.50	0.66	3.17	9200	770	5145	1985	1860		

[1] Periods XV to XXII in table 2.

[2] Calculated from Sherman's tables ('27).

[3] Calculated from analyses of diets and individual foodstuffs made in this laboratory.

of total base excretion in the feces though there was an increased retention of total base because of a greater reduction in urinary excretion.

The addition of sodium chloride to the diet (10 to 15 ˙gm. per day, periods XVIII–XXX) also had minimal effects on the calcium excretion, possibly elevating it a little in the urine. From the total base analyses it is clear that this large addition of sodium chloride to the diet did not increase fecal total base excretion.

It, therefore, appears clear that fecal volume, or a high sodium chloride or fat intake have negligible effects on fecal excretion of calcium and phosphate. A high urea ingestion has a greater effect on urinary calcium excretion than any of these, but that effect is not of large proportions.

<center>DISCUSSION</center>

The evidence introduced in this paper demonstrates the relative stability of calcium absorption and excretion, for this remains remarkably constant in spite of great variations in factors which might be expected to alter its metabolism. The patients suffering from parathyroid over-activity greatly need calcium to counteract the great demands on their bones for this base. Yet there is no evidence that their excessive parathyroid secretion uniformly increases the absorption from the bowel. These observations also indicate that an excellent organic solvent like urea has no apparent influence in aiding calcium absorption nor does voluntary constipation or diarrhea influence its excretion. Yet calcium absorption from the intestine can be markedly improved by some unknown influence (G.M., table 1).

A similar independence is demonstrated in regard to kidney excretion of calcium. It must be recalled that calcium differs from many other bases in the body in that there are vast stores in the bones which can be readily replenished or depleted according to body needs (Bauer, Aub and Albright, '29). In this regard its metabolism resembles that of carbohydrates. One might expect that these large stores of readily

available calcium would cause large variations in the amount of its excretion in the urine. But in these observations it is clear that urea increases the calcium excretion only moderately, and· this quite independently of whether there is a marked diuresis or not, or whether there is an accompanying rise of serum calcium. Furthermore, in the hyperparathyroid patient who had the smallest calcium stores we have ever seen, a high blood calcium was not accompanied by the usual high urinary excretion. There are, therefore, factors other than kidney threshold alone which influence urinary calcium excretion—evidence for which is also seen in the normal blood levels but elevated excretion found in thyroid and pituitary over-activity (Aub, Bauer, Heath and Ropes, '29; Tibbetts and Aub, '37).

CONCLUSIONS

These observations justify the following conclusions:

1. Parathyroid over-secretion has previously been shown to have no influence on fecal calcium excretion. These observations indicate that it has no consistent effect on intestinal absorption of calcium.

2. An excellent organic solvent like urea does not influence calcium absorption from the intestine. Urea ingestion, however, does elevate the blood calcium level somewhat in exophthalmic goiter and hyperparathyroidism, and increases urinary calcium excretion in all cases. This increased excretion is independent of diuresis.

3. Calcium excretion in normal subjects is independent of voluntary constipation, cascara catharsis, or the ingestion of agar, large amounts of fat or of sodium chloride.

4. Calcium excretion is, therefore, independent of many factors which might be expected to affect it, and in health remains at a remarkably constant level considering the vast available store in the bones.

5. That some factor greatly increases calcium absorption from the intestines is again made obvious in these observations, but its nature is still unknown.

METHODS

The analytical methods used were: Calcium by the method of Fiske and Logan ('31); phosphorus according to Fiske and Subbarow ('25); total base by the Fiske method ('22); chloride by that of Van Slyke ('23); nitrogen by the Kjeldahl; and urea by the urease method described by Folin ('34).

CASE HISTORY

M.T. (C. P. Huntington Memorial Hospital no. 33-619; M.G.H. no. 330046; series, case II; Path. no. 33-2429 and no. 33-4429), female, 53 years of age. For 13 years she had had bone pain which caused her to limp. In the last 4 years she had had operations for 1) a giant-cell tumor of the upper jaw, 2) and one of the nose, 3) uterine suspension, 4) fractures of three bones (3 years ago), 5) curetting cyst of the right tibia (1½ years ago), and 6) spontaneous fracture of the right femur and of the right tibia and fibula. On physical examination her condition appeared critical due to great asthenia and great anorexia and vomiting.

P.E. A pathetic looking woman with squashed-in torso who has to assume a sitting position because of her fractures and marked upper dorsal spinal kyphosis. She is incapable of moving herself. The only two remaining teeth are very loose and carious. There were a few inconstant rales, and asthmatic breathing in the lungs. Heart appeared normal. Liver edge just palpable. No peripheral edema. Blood pressure 140/75.

Laboratory findings. Urine, with a specific gravity of 1.016, was normal except for a faint trace of albumin, and hyaline and granular casts once. Wassermann, Kahn and Hinton tests were negative. Phenolsulphonphthalein test—intramuscular: first hour, 60 cc. = 15%; second hour 180 cc. = 35%; total = 50%. Non-protein nitrogen = 33 mg.%. Feces were normal. Blood was normal—no anemia. X-ray examination of the whole skeleton showed extensive loss of lime salts and cyst formation in practically all long bones. There were spontaneous fractures of the neck of the left femur and of both the left and right tibiae and fibulae, and of the right clavicle. There was a small substernal tumor mass which pushed the trachea, but not the esophagus, to the right.

After our observations, two operations were performed at the Massachusetts General Hospital by Doctors Churchill and Cope, who found a parathyroidoma composed largely of

chief and giant multinucleated cells. Her recovery has been remarkable. She now feels practically well and is doing a full day's work.

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DIETARY PRODUCTION OF THE SYNDROME OF DEFICIENCY IN VITAMIN B₆[1,2,3]

NELLIE HALLIDAY AND HERBERT M. EVANS

Institute of Experimental Biology, University of California, Berkeley

ONE FIGURE

With the knowledge that vitamin G (B$_2$) is multiple in nature, it has become increasingly important to standardize methods by which the various factors may be determined quantitatively. The present paper describes the steps we have taken and the results we have obtained in an attempt to prepare a diet which will induce vitamin B$_6$ deficiency with regularity and uniformity.

The Bourquin-Sherman basal diet ('31) for determining vitamin 'G' carries an 80% alcohol extract of whole wheat as a source of vitamin B (B$_1$). Cereals have been shown to carry a varying but in general a considerable amount of vitamin B$_6$. About 25% of the vitamin is extracted by the alcohol and from the whole wheat extract prepared in this laboratory (as we have recently reported ('37)) sufficient flavin as well as the 'filtrate factor' of Lepkovsky and Jukes ('35, '36 a, '36 b, '36 c) and Lepkovsky, Jukes and Krause ('36)

can be extracted at the same time to allow a slow but continuous rate of growth. It is interesting to consider, however, that in the early work when the method was standardized, as well as in later work by Sherman and collaborators ('31, '33, '35), by Booher ('33, '34), by Booher et al. ('34), and by Halliday (in East Lansing, Michigan) ('31, '32) dermatitis was induced with a high degree of regularity, particularly when precautions were taken to prevent coprophagy. György ('35) noted that different lots of wheat varied as to their vitamin B_6 value. In this laboratory we have been unable to induce dermatitis on the Bourquin-Sherman diet, due undoubtedly, to a difference in the whole wheat.

With the availability of crystalline or highly potent concentrates of vitamin B (B_1), the whole wheat extract may be eliminated. Theoretically a basal diet deficient in all B factors supplemented by B_1 and flavin with or without the 'filtrate factor' should induce vitamin B_6 deficiency.

The Sherman-Spohn ('23) diet is known to be free of vitamin B and has been considered to be free of all B factors. The composition is: casein (extracted twice with cold 60% alcohol and once with cold 95% alcohol) 18%; Osborne and Mendel salt mixture ('19) 4%; filtered butter fat 8%; cod liver oil 2%; and cornstarch 68%. Our investigations concerned modifications in the carbohydrate, casein and fat content of this diet.

The question of carbohydrate has received much attention and varying results have been reported. Hogan and co-workers ('34, '35, '36 a, '36 b) have found an antidermatitis factor in cornstarch. Bender et al. ('36) demonstrated protection from dermatitis and increased growth with dextrin rather than sucrose in the diet. Chick and co-workers ('35) reported a more regular incidence of dermatitis when maize sugar replaced starch. On the other hand, György ('35), Birch, György and Harris ('35), and Birch and György ('36) have used rice starch extensively in their work on vitamin B_6 and flavin. Jansen, Westenbrink and Boeke ('35) could not induce normal growth on sucrose diets when supplemented

with yeast extract, and preferred rice starch in studies on vitamin B_6. Ariyama ('36) concluded that rats receiving 60% sucrose required, in addition to B, flavin and a concentrate carrying B_6, some factor found to be present in yeast or the alcohol extract of ox liver, lettuce or spinach. He considered the factor was not a member of the B complex. Jackson ('30) found that fatty livers developed in rats receiving an 80% sucrose, fat-free diet supplemented by yeast, wheat germ, cod liver oil, and, in some cases, 10 drops of lard. Sucrose has been used in this laboratory for a number of years when a high degree of purity has been desired in fat-free diets as well as in work on the B vitamins.

Various methods have been used for purification of casein. The English workers have used the 'light white casein' or the 'Glaxo AB' casein without further purification. Supplee and co-workers ('36 a and '36 b) use a casein purified by salt elutions. Elvehjem and co-workers ('36) prepare their casein by precipitation with hydrochloric acid, dissolving in ammonia and reprecipitating with acid. Lepkovsky and Jukes wash commercial casein for 1 week with acidified water followed by one washing with dilute alcohol. Hogan and co-workers ('32, '36 a, '36 b) extract their casein continuously for 1 week with 70% alcohol in a Soxhlet apparatus. We observed that more fluorescent material (flavin?) was extracted by boiling 95% alcohol than by cold 60% alcohol. We therefore extracted the casein several times with hot 95% alcohol until no fluorescence could be demonstrated when the alcohol extract was examined with 'black light,' as described by Supplee and co-workers. This treatment was followed by two extractions with cold 60% and one with cold 95% alcohol.

We made up three diets: 787 was the original Sherman-Spohn diet; 787-A was this diet modified by the inclusion of sugar in place of cornstarch; and 787-B was the diet modified by the inclusion of the more highly purified casein.

In order to evaluate the results we scored the rats with respect to the severity of symptoms as follows: + denotes mild symptoms. The fur, ears and forelegs are blood stained and growth has ceased. ++ denotes a more severe condition.

The ears are swollen, there is a sore area around the nose and mouth, and the feet are red and swollen. At this stage, the animals have begun to lose weight. +++, in this stage the eyes are encrusted, all four feet are raw and the ears are swollen and hard. The nose and mouth are fairly sore. ++++ denotes the final stage. The entire area around the nose and mouth is raw, the feet and forelegs are edematous and sore, the eyes are entirely closed, the ears have sometimes broken off and there is a bloody urine. The animals have lost 10 to 20 gm. in weight.

In spite of the care in the extraction of the casein, we have not been able to induce the denuding on the back and face as described by Hogan, by György and others, as symptomatic of flavin deficiency. Often the abdomen is denuded, but this appears to be due to irritation. We have, however, found denuding of the face in animals which have survived rather longer although they have received flavin. Supplee and co-workers have discussed the difficulty encountered in freeing casein of flavin. They consider the flavin to be bound to casein as a prosthetic group.

EXPERIMENTAL

Twenty-one-day-old female rats weighing 40 to 50 gm. were evenly matched and divided into three groups to test the three diets. A vitamin B concentrate was supplied as a separate supplement. When tested against the Chase and Sherman ('31) vitamin B deficient diet, 200 γ of this material induced a gain of approximately 1.5 gm./day, and this amount was fed to the test rats. (György has found that with too low an intake of B there is a delayed incidence of dermatitis.) Within a few days the rats receiving the sugar diet developed severe diarrhoea which appeared to make them less standard as test animals. It was not possible with the sugar diet to obtain any figure as to food consumption. The animals receiving diets 787-A and 787-B developed dermatitis at about the same time and of about the same degree of severity. Due to these considerations diet 787-B was used extensively for several months. In table 1 are shown the results. The sur-

TABLE 1

A comparison of the incidence of dermatitis, severity of symptoms, and survival periods of rats which received the diets and supplements as described

DIET AND SUPPLEMENTS	NUMBER OF RATS	AGE AT FIRST SYMPTOMS (days)	RANGE (days)	MEAN DEVIATION	S.D.	P.E.	COEFFICIENT OF VARIATION (%)	SEVERITY OF SYMPTOMS, NUMBER OF PLUSES	SURVIVAL PERIOD DAYS ON DIET (days)	RANGE (days)	MEAN DEVIATION	S.D.	P.E.	COEFFICIENT OF VARIATION (%)	SURVIVAL PERIOD AGE (days)	RANGE (days)	MEAN DEVIATION	S.D.	P.E.	COEFFICIENT OF VARIATION (%)
Diet 787 + B_1 only	7	64.7	(54–85)	±34.0				2.9	131	(68–173)	±34.0				152	(89–194)	±34.0			
787-A + B_1 only	5	54.4	(50–57)	± 2.0				3.2	89	(39–120)	±25.2				110	(60–141)	±25.2			
787-B + B_1 only	60	57.3	(32–105)	±11.2	14.7	1.3	25.8	3.3												
Same + B_1 and flavin with or without other supplement	38								105	(35–180)	±28.0	33.3	3.6	31.9	125.9	(56–201)	±26.5	33.7	3.6	26.8
Diet 793 + B_1 only	17	54.1	(33–69)	±10.8	12.6	2.1	23.2	3.4	86.6	(60–118)	±18.0	20.3	3.3	23.4	107.6	(81–139)	±18.0	20.3	3.3	18.8
+ B_1 and flavin with or without other supplement	23	54.5	(44–63)	± 4.9	5.8	0.8	10.6	3.7	68.2	(45–89)	± 8.2	10.8	1.5	15.9	89.2	(66–110)	± 8.2	10.8	1.5	12.2
Diet 794 + B_1 only	33	56.6	(44–64)	± 5.5	6.3	0.7	11.1	3.5	62.7	(36–87)	±11.1	13.5	1.6	21.5	83.6	(57–108)	±11.1	13.5	1.6	16.1
+ B_1 and flavin with or without other supplement	10	46.8	(40–55)	± 2.6	3.8	0.8	8.2	3.7	65.1	(55–81)	± 5.1	7.3	1.6	11.2	86.1	(76–102)	± 5.1	7.3	1.6	8.4
+ B_1 and flavin with or without other supplement	13	47.1	(41–55)	± 2.5	1.2	0.2	2.5	3.9	64.4	(50–76)	± 6.9	8.5	1.6	13.2	85.4	(71–97)	± 6.9	8.5	1.6	13.2

vival period of rats receiving diet 787 was very much greater than of rats receiving the other two diets due undoubtedly to difference in the casein. Of sixty rats which received diet 787-B plus B, twenty-two were later used for curative experiments. Survival periods of the remaining thirty-eight animals are shown. Only three rats which received diet 787-B plus B failed to show any symptoms and they were eventually killed. Their results are not included in the table. It is interesting to note that seventeen rats which were used for experimental work and received in addition to vitamin B, flavin with or without a further supplement low in vitamin B_6 survived only 86.6 ± 18.0 days on the diet. They thus showed a shorter survival period than those which received B supplement only, and confirmed the findings of Chick ('35) and co-workers on this point.

In all experimental work rats were given at weaning the basal diet plus vitamin B, and in some cases B and flavin until growth ceased and symptoms of about 1+ degree of severity were induced. This required from 4 to 5 weeks on an average for diet 787-B, and about 3 to 4 weeks for the sugar diets used in later work to be described.

It was still felt that because of its relatively greater purity sugar would be preferable to starch. We therefore investigated the effect of lowering the fat content, in an effort to reduce the incidence of diarrhoea. György, and Bender and co-workers had both reported some fat sparing action with respect to vitamin B_6.

Diets 793 and 794 (the latter carrying casein extracted according to the Hogan technic) were prepared. The composition was as follows: casein 18%, salts 4%, filtered butter fat 3%, cod liver oil 2% and sucrose 73%.

From table 1 it is seen that results with these two diets were closely similar. There was not the large difference between animals receiving additional supplements and those receiving vitamin B only as was seen in the earlier work with diet 787-B. There was 100% incidence of dermatitis of nearly 4+ severity. There were no spontaneous cures. However,

there was considerable diarrhoea, particularly in the later stages.

Lepkovsky, Jukes and Krause ('36 d) find that on their purified diet, supplemented by vitamin B and flavin, most rats die without manifesting symptoms. Addition of the 'filtrate factor' is necessary before dermatitis develops. It would hardly seem that our diet could carry any of the 'filtrate factor.' It is postulated, therefore, that the growth stimulus furnished by this added factor might cause the dermatitis to become severe, in the experiments described by these workers. In a recent paper Elvehjem ('36) and co-workers describe a series of diets used in preliminary work on their liver growth factor. The diets carry starch, dextrin or sugar, and from 7 to 14% fat. Animals given these diets supplemented by vitamin B failed to grow, and the authors state that the rats failed to show any symptoms which might aid in determining the specific deficiency. György found that the average time of appearance of symptoms was about 7 weeks. In these experiments the rats were depleted 21 to 25 days and the greater majority of them, unless protected, died within the next 5 weeks.

It seems, therefore, that diets 793 and 794 can be depended upon for uniform results. If it is desired to obtain food records, diet 787-B can be used.

We cannot explain why we found it possible to induce the specific dermatitis on a cornstarch diet. One of us has visited Doctor Hogan's laboratory, and, as far as it is possible to judge, the dermatitis he induces and cures with wheat germ oil is identical with the conditions we describe. We have not been successful as yet in curing dermatitis with wheat germ oil in this laboratory, but these experiments are still under way.

Itter and co-workers ('35) correlated alopecia with the absence of the sulfhydryl group, and found that feeding of cysteine HCl led to cures. Prunty and Roscoe ('35) showed that purified casein might be deficient in cystine, with the result that the growth rate was lessened. They could not cure

the dermatitis symptomatic of B_6 deficiency by the addition of cystine, however.

In order to ascertain whether or not cystine could be a limiting factor in our results, we prepared a modification of diet 787-B in which 0.25% cystine replaced that amount of starch. A paired feeding experiment was carried out, the animals which received the non-supplemented diet acting as

Average growth curves of rats receiving diet 787-B + B_1 concentrate alone (I), or II this diet plus dried liver = 0.25 gm fresh liver daily.

Average growth curves of rats receiving cystine-containing diet + B_1 alone (III), or IV this diet + dried liver = 0.25 fresh liver daily.

Gain or loss with respect to the cystine diet.

Average gains of animals receiving diet 787-B + B_1 concentrate and liver when plotted as gains above that made by negative controls.

Average gains of animals receiving cystine-containing diet + B_1 concentrate and liver when plotted as gains above that made by negative controls.

Gain or loss with respect to the cystine diet.

Figure 1

controls. Prunty and Roscoe had stated that when a low level of vitamin 'B$_2$' was fed, addition of cystine improved the growth rate. We therefore fed (in addition to vitamin B) dried liver ∽ to 0.25 gm. fresh material to the experimental animals of the two groups. Figure 1 shows the results. There was definitely no increase in growth as a result of the cystine addition. Furthermore, rats which received only vitamin B in addition to the cystine diet developed dermatitis

somewhat earlier than did those of the non-supplemented diet group.

Using the method of Prunty ('33) for determination of cystine we found 0.34%, 0.24% and 0.20% for the cystine content of the unextracted casein, the casein used in diets 787-B and 793, and that used in 794, respectively. Thus the cystine content is lowered but, from our experimental results, it would seem that neither the loss in weight nor the severe dermatitis produced could be due to cystine deficiency.

The occurrence of diarrhoea among the animals in this work appears to be somewhat unique. Doctor Hogan has used a low fat, high sugar diet for some years and has not encountered the phenomenon. Lepkovsky and Jukes ('36 c), using 59% sugar and 10% lard in their diet state that diarrhoea is a characteristic symptom of flavin deficiency. Many of our rats, however, developed the condition while receiving flavin. It seems that further work will be required to answer this question.

We did not use the Page ('32) anti-coprophagy harness in these experiments. Had we done so, it is quite probable that the variations which occurred within groups would have been materially lessened.

CONCLUSIONS

Experiments were carried out in an attempt to develop a basal diet for rats which would induce the syndrome of vitamin B₆ deficiency with regularity and uniformity.

The effects due to the method of extraction of the casein, the type of carbohydrate and the percentage of fat in the diet were studied.

From results with large numbers of rats it is concluded that a diet carrying high sucrose, low fat, and casein extracted with both boiling 95% alcohol and cold 60% alcohol can be depended upon to produce uniform results as to severity of symptoms and length of survival period of rats. A system of scoring the symptoms was developed which permitted quantitative comparison of the animals in the various groups.

Although the method of preparation of the casein lowered its cystine content, this did not appear to be a factor in our results.

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THE FOOD INTAKE OF YOUNG RATS HELD AT NEARLY CONSTANT BODY WEIGHT BY RESTRICTION OF THE DIETARY PROTEIN [1]

C. M. JACKSON

Department of Anatomy, University of Minnesota, Minneapolis

ONE FIGURE

It is well known that growth in young animals can be retarded or suppressed by restriction of the dietary intake of protein. The morphological changes, especially in the organ weights, under such conditions were studied by Limson and Jackson ('32) and Jackson ('36). The present report deals with the food intake during the latter experiment. The data include the amount of protein (in the form of yeast-wheat germ mixture) required to maintain the body weight, and the corresponding voluntary intake of basal diet when the protein intake is thus restricted.

MATERIAL AND METHODS

The albino rats used in this work are from the standardized colony maintained for nutritional experiments in the Institute of Anatomy. They were originally derived from the Wistar experimental strain. The test rats used in the present study include 35 males and 32 females (with 3 in addition to the 33 males and 31 females reported by Jackson, '36). Large groups of animals are desirable on account of individual variations, as shown for metabolism by Benedict and

MacLeod ('29). All the rats were weaned and weighed on the twenty-first day of age, when the experiment began.

The test rats were then placed in metal individual cages with wire-net floors, on the protein-deficient diet used in the two preceding studies. The basal diet mixture, fed ad libitum, included sucrose 75%, lard 20%, and salt mixture (McCollum 185) 4.5%. The consumption was measured by weighing the initial amount placed in the cage, and later the residual amount left in the food cups, together with the scattered food carefully collected from a paper sheet placed beneath the floor of the cage. City tap water was also supplied ad libitum from bottles in the cages. The accessories, fed separately to each rat daily, included 2 drops of cod liver oil (Patch), and a variable amount of mixture of equal parts by weight of dried brewer's yeast (Northwestern pure dehydrated) and wheat germ (Russell Miller Milling Co.). As explained by Limson and Jackson, this diet provides adequate nutrition for all the known factors except protein, which is represented by the yeast-wheat germ mixture. This mixture was fed in varied amounts from day to day, regulated so as to hold the body weight as nearly constant as possible during the experimental period of 15 weeks, that is, from 3 to 18 weeks of age.

During the period of growth repression, the test rats were kept in a constant temperature room at about 26°C. (79°F.) with a daily variation of less than 1°C. According to Benedict and MacLeod ('29), this is slightly below the critical temperature for the rat. While in this room, the rats were observed and weighed daily.

<center>OBSERVATIONS</center>

The general appearance of the rats during the experiment was reported by Jackson ('36) and need not be repeated here.

Body weight. As shown in figure 1, it was not possible to hold the body weight entirely constant. A slight loss was unavoidable during the first week of the test, while the rats were becoming adjusted to the new diet. Thus the average of the thirty-five males decreased from the initial weight of 49.0 gm.

to 46.1 gm. at the end of the first week. The loss was soon recovered, and the weight remained fairly constant between 50 and 51 gm. from the sixth week onward. Similarly the average weight of the thirty-two females decreased from 47.2 to 44.2 gm. during the first week, then recovering to a level between 48 and 49 gm. which was maintained from the sixth
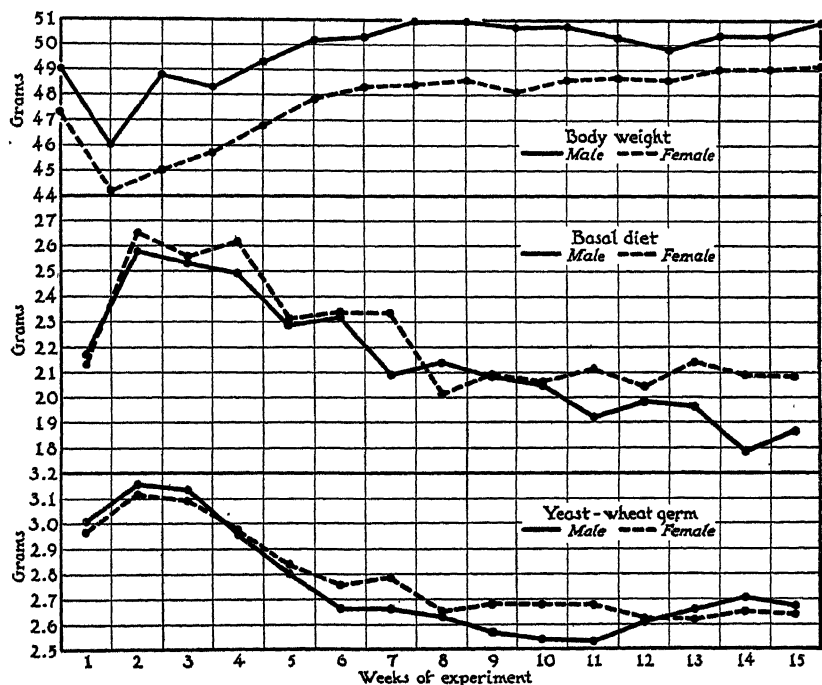
Fig. 1 Curves showing the changes in average body weight for each sex by weeks during the period of the experiment; with corresponding changes in the consumption of basal diet (ad libitum) and of yeast-wheat germ mixture (regulated to hold the body weight nearly constant).

week onward. The figures for body weight for the various weeks in table 1 differ slightly in that they represent the estimated averages for the corresponding weeks, instead of the weights at the beginning or end of the week. The mean of the weekly average male weights (49.81 gm.) was about 2 gm. above that of the females (47.65 gm.). This represents

the average body weight for each sex during the entire experimental period.

Protein requirement. As is evident in figure 1, the amount of the yeast-wheat germ mixture required to maintain the body weight under these conditions showed a characteristic change during the course of the experiment. During the first week (period of adjustment to the diet) the average intake

TABLE 1

Weekly average body weights and intakes of food per gram of body weight

	THIRTY-FIVE MALES				THIRTY-TWO FEMALES			
WEEK	Body weight	Intake per gram body weight			Body weight	Intake per gram body weight		
		Basal diet	Yeast-wheat germ	Total [1]		Basal diet	Yeast-wheat germ	Total [1]
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	47.5	0.456	0.0633	0.534	45.8	0.466	0.0649	0.546
2	47.5	0.542	0.0663	0.623	44.6	0.595	0.0700	0.681
3	48.5	0.522	0.0645	0.600	45.4	0.565	0.0681	0.648
4	48.8	0.511	0.0605	0.586	46.3	0.566	0.0642	0.645
5	49.7	0.459	0.0563	0.529	47.3	0.488	0.0600	0.563
6	50.3	0.462	0.0530	0.529	48.1	0.463	0.0574	0.535
7	50.7	0.413	0.0525	0.479	48.3	0.483	0.0576	0.556
8	51.0	0.419	0.0516	0.485	48.5	0.414	0.0546	0.483
9	50.9	0.409	0.0505	0.473	48.3	0.431	0.0555	0.501
10	50.7	0.404	0.0501	0.468	48.3	0.426	0.0555	0.496
11	50.5	0.381	0.0501	0.445	48.6	0.435	0.0552	0.504
12	50.0	0.396	0.0522	0.462	48.6	0.422	0.0539	0.490
13	50.0	0.391	0.0532	0.458	48.7	0.440	0.0538	0.508
14	50.3	0.356	0.0539	0.424	49.0	0.426	0.0541	0.494
15	50.7	0.368	0.0527	0.435	49.0	0.424	0.0539	0.492
Average	49.81	0.433	0.0554	0.502	47.65	0.470	0.0586	0.543

[1] Total includes the cod liver oil (2 drops daily per rat).

of the mixture per rat was about 3 gm. Beginning with the second week, the consumption decreased steadily from over 3.1 gm. to about 2.7 gm. in the sixth week. This decrease in protein requirement corresponds to the period of slight increase in average body weight. Thereafter, during the period of nearly stationary body weight, the requirement of yeast-wheat germ likewise remained nearly constant at the comparatively low level of 2.6 or 2.7 gm. per week, or slightly

below 0.4 gm. per rat daily. The corresponding data, figured on the basis of the weekly intake per gram rat are shown in table 1. As was noted by Limson and Jackson ('32), the actual protein component probably does not exceed one-third of the yeast-wheat germ mixture.

Another feature of interest is the apparent sex difference in the required protein intake. The difference in the total intake is not striking, as shown in figure 1, although the female intake appears higher during the greater part of the period. When the difference in body weight is taken into account, however, as shown in table 1, the average protein intake per gram rat appears invariably higher in the female. The difference is not very great (average 5.8%) but the constancy makes it probable that it is significant. The usual statistical formula for the probable error of the difference in means is not appropriate here, because we do not have 'normal distribution' of the variates. More complicated methods of measuring the significance of the difference were not attempted. We may, however, conclude that the males are apparently able to maintain their body weight with a relatively lower protein intake.

Basal diet intake. The amount of basal diet voluntarily consumed by the rats runs closely parallel to their restricted protein intake, as shown by the general course of the curves in figure 1. After an initial increase in the second week (following the period of adjustment in the first week), there is a steady decrease in the basal diet consumed during the first half of the experiment, followed by a more nearly stationary period in the second half.

The ratio of the basal diet intake to the yeast-wheat germ mixture during the successive weeks remains remarkably constant. From the data given in table 1, the percentage of yeast-wheat germ intake to basal diet may be calculated for either sex, on the basis of consumption per gram of body weight. The percentage of yeast-wheat germ in comparison with total food intake (basal diet plus yeast-wheat germ and cod liver oil) is slightly lower but similarly constant, averaging close to 11% for each sex (general range 10% to 12%).

While the ratio of protein to voluntary total diet thus appears similar in each sex, there is an apparent sex difference in the amount of basal (and total) diet consumed. As shown in figure 1, the average female intake of basal diet exceeds that of the male in every week excepting the first and eighth. This difference is still more apparent when calculated on the basis of intake per gram body weight, from the data in table 1. The average weekly intake of basal diet per gram body weight in the females (0.470 gm.) exceeds that of the males (0.433 gm.) by 8.5%. The percentage difference for total dietary intake is about the same. Here again, as above mentioned for the yeast-wheat germ, the usual statistical formula for probable error of the difference cannot properly be employed, since this requires a 'normal distribution' of the variates. More complicated statistical methods were not attempted, but even without these the significance of the sex difference can scarcely be doubted.

Dietary calories. The energy value of the various dietary components consumed was calculated at 4 calories per gram for the sucrose and yeast-wheat germ, and 9 calories per gram for the lard and cod liver oil (assuming 2 drops = 0.1 gm.). The changes in energy from week to week necessarily correspond to the weights of the diet consumed. The total dietary intake by the male rats during the 15 weeks' period averages 1807.72 calories per rat. The corresponding amount for the female rats averages 1872.94 calories, or 3.6% greater. Since the body weight of the female rats is lower, the caloric value of their diet consumed per unit of body weight is correspondingly greater. Calculated on a relative basis, the energy value of the total diet consumed averaged 0.346 calorie daily per gram body weight for the male rats, and 0.374 calorie for the females. This amounts to an excess of about 8% for the females, corresponding to the difference in weight of diet consumed during the period.

DISCUSSION

The well-known decrease in the general dietary requirement for young animals during the course of maintenance experiments might be explained in different ways. The most important factor is probably the associated decrease in basal metabolism resulting from inanition. This decrease has recently been shown in fasting rats by Benedict and Fox ('34) and in stunted young rats by Horst, Mendel and Benedict ('34). The latter observed a lowered basal metabolism (heat production) when growth was repressed by low protein diet, as well as during underfeeding tests. They also noted that the lowered metabolism occurs in spite of increased activity. An increase in the spontaneous activity of the stunted rats was likewise evident in the present experiment.

Since in the present investigation the voluntary food intake was reduced in proportion to the limited protein, it is probable that general caloric deficiency may be another important factor in the associated repression of growth. This conclusion is supported by the data of McCay, Crowell and Maynard ('35), who arrested growth by general underfeeding of a somewhat different diet. They found that to maintain the body weight of rats at 50 gm. required 0.286 calorie per gram rat daily for the males and 0.304 calorie for the females. This is not far below my average of 0.346 calorie consumed by the males and 0.374 calorie by the females. My results are therefore in harmony with the conclusion of R. W. Jackson ('29) that a rat eating ad libitum of a diet limited in one essential factor (so as to cause approximate weight maintenance) will ingest very little more energy than can be used advantageously under these conditions.

In experiments extending over a considerable period of time, the age factor may also be contributory in reducing the nutritive requirement. In general, the heat production in young rats appears normally to decrease with age, as shown by Davis and Hastings ('34), Sherman and Campbell ('35), and Sherwood ('36). McCay, Crowell and Maynard ('35) similarly found that at successive stages of greater age and

body weight there is a quite regular decrease in the calory requirement for maintenance per unit of body weight. As is pointed out by Johnson, Hogan and Ashworth ('36), the relative protein requirement likewise diminishes normally with age.

Numerous workers have discovered sex differences in the nutritional requirements of rats. Horst, Mendel and Benedict ('34), observed that, in general, adult male rats have a higher basal metabolism, which is in accordance with the usual finding in other species. However, Davis and Hastings ('34) and Sherwood ('36) could find no appreciable sex difference in basal metabolism, especially in younger rats. Wang ('25) observed a relatively greater food intake in the female rats. Mitchell and Carman ('26) and Morris, Palmer and Kennedy ('33) concluded that the female rat consumes relatively more energy per unit gain in body weight. McCay, Crowell and Maynard ('35) similarly noted that the female rat requires more calories for maintenance. In the absence of any evidence for higher basal metabolism in the female rat, the workers have usually ascribed the higher energy intake of the female to greater activity. Apparently the only direct evidence of such activity was published by Slonaker ('12), whose conclusions were based upon a female rat showing greater activity than the males after the fourth month of life. Limson and Jackson ('32) observed no appreciable sex diference in the intake of food under conditions similar in general to the present experiment, but at ordinary fluctuating room temperatures instead of constant temperature. The present results support the conclusion that the energy requirement is relatively higher in the female, whatever may be the significance of this phenomenon. It furthermore appears that this sex difference applies not only to the total diet, but also to the protein (yeast-wheat germ) requirement for maintenance.

SUMMARY

Thirty-five male and thirty-two female albino rats (Wistar strain) were maintained for 15 weeks at body weight of about 50 gm. by regulated amounts of protein (yeast-wheat germ) intake. A diet otherwise adequate for normal nutrition was fed, including 2 drops of cod liver oil daily with basal diet ad libitum. The room was held at nearly constant temperature (26°C.).

Under these conditions, the required (limited) intake of yeast-wheat germ mixture increased slightly during the first week (period of adjustment). Beginning with the second week, the consumption decreased steadily from an average of over 3.1 gm. per rat to about 2.7 gm. in the sixth week, remaining nearly stationary thereafter.

The corresponding voluntary intake of basal diet ran closely parallel to the limited protein intake, the average weight of yeast-wheat germ remaining nearly constant at about 11% of the total diet (including accessories). The calories of the total diet appeared slightly above the energy requirement for maintenance.

The data indicate a slight but probably significant sex difference in the food intake of the rats under these conditions. The male rat apparently requires relatively less protein (yeast-wheat germ) for maintenance, and shows likewise a relatively smaller intake of the basal and total diet.

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VITAMIN E

GUSTAV J. MARTIN

Chemistry Department, College of St. Teresa, Winona, Minnesota

Due to greater interest in the anti-sterility properties of vitamin E, comparatively little attention has been given to its growth effect. Attempting the cure of male sterility, Evans and Burr ('27) noted that although cures were not effected, the administration of vitamin E was marked by an increase in the rate of growth. Subsequently, Evans ('28–'29) found that animals of both sexes reared on vitamin E-free diets showed, after the eighth month of life, but not before, a consistently poorer rate of growth than did rats receiving the same diet supplemented daily by a few drops of wheat germ oil. The same effect was observed with the unsaponifiable portion of this oil. Blumberg ('35) reported the use of a highly purified diet which was deficient in vitamin E. On this diet, normal early and middle growth failed to occur. Female rats showed subnormal growth in the first 5 weeks on the diet, and had plateaued in 18 weeks. In distribution and general properties, the growth principle resembled vitamin E. Some evidence obtained favored the view that wheat germ oil contained an unrecognized factor essential for optimal growth. Olcott and Mattill ('36) reported no improved growth in rats fed stock rations as compared to those on highly purified vitamin E deficient diets. The deficiency of vitamin E in the purified diet was established by proved sterility at 130 days. They conclude that the early growth effect of other observers could not be due to the vitamin E content of the supplements used.

Coward and co-workers ('29) and Guha ('31) produced a deficiency disease using special casein, but they did not prove that the curative principle was not vitamin E. Mason ('28–'29) believed that the beneficial effect of lettuce on growth was not due to its vitamin E content alone. The water-soluble early growth factor in liver, reported by Mapson ('32), would seem to be independent of any possible growth effect of vitamin E. Blumberg ('35) noted in the case of two male animals fed the experimental diet but with the addition of a concentrate (equivalent to 2 gm. per week of wheat germ oil—an amount in excess of the fertility requirement) that the growth curves were subnormal at the end of the twenty-eighth week, despite proved fertility by the mating test. This observation led to the investigation of the possible bipartite nature of the non-saponifiable matter of wheat germ oil with regard to the growth and anti-sterility effect attributed to it.

TECHNIC

The purified diet used was as follows:

Basal diet

	%
Casein (ether and alcohol extracted)	20
Yeast (ether extracted)	10
Salt mixture no. 185 (McCollum et al., '28)	4
Sucrose (commercial)	66

Supplements

Carotene—An ethyl laurate solution, fed at 2 drops per day equivalent to 0.08 mg. of carotene per rat per day. Solution prepared fresh every week and stored under carbon dioxide.

Irradiated ergosterol—An ethyl oleate solution of the resin, incorporated in the diet at 30 drops per kilogram, equivalent to 6 drops per kilogram of viosterol 250 D.

Ethyl linolate—A distilled oil, fed at 3 drops per day equivalent to 65 mg. per rat per day.

The details of preparation and feeding are as described by Blumberg ('35).

EXPERIMENTAL

A group of 58 rats, 26 males and 32 females, were placed on the basal diet with the supplements indicated. The animals were selected at weaning and weighed from 35 to 50 gm. each. These animals showed a slowing of growth at the tenth week and had plateaued at decidedly subnormal weights by the eighteenth week. The average plateauing weight of the females was 167 gm. (On stock diet females reach 190 to 210 gm.) which corroborates the observation of Blumberg ('35). For the males, the average weight when plateaued was 269 gm., whereas on stock diet males reach 350 to 450 gm. Each of the vitamin supplements was checked to assure optimal feeding levels by doubling the amounts and observing no additional response in animals plateaued on the diet as described. The possibility of excess dosage was checked by reducing the amount fed to one-half. Blumberg ('35) reported the completeness of this diet with respect to all known factors except vitamin E. When the sterility of the animals was proved by demonstrating non-motile sperm in the males and resorption of foetus in the females, supplements of variously prepared wheat germ oil concentrates were added.

A preparation of the unsaponifiable matter of wheat germ oil was prepared by hot saponification (2 hours at 80°C.) following the procedure of Burr and Burr ('30). The test animals, eight females and four males, showed weight gains averaging 27 gm. for the females and 26 gm. for the males in a period of 6 weeks on this supplement. The concentrate was fed at a level equivalent to 2.5 gm. of wheat germ oil per week. The females of each group were tested for fertility after they had been used to detect growth-promoting action of concentrates. The successful delivery of normal young when mated with a stock diet male was taken as evidence of the presence in the concentrate of ample vitamin E for protection against sterility. The growth response of these animals to the vitamin E concentrate of wheat germ oil confirms the observations of Blumberg ('35).

TABLE 1

Weight responses of rats to vitamin E concentrates; 6 weeks' feeding

(rats used were 18 weeks old and had previously plateaued on the basal diet)

SUPPLEMENT	ANTI-STERILITY POTENCY	WEIGHT GAIN IN 6 WEEKS IN GRAMS							
		Females				Males			
		Number	Average	Maximum	Minimum	Number	Average	Maximum	Minimum
Controls: vitamin E free basic diet	Negative	8	3	5	— 1	2	— 20	— 26	— 14
Ether soluble non-saponifiable matter of wheat germ oil = 2.5 gm. wheat germ oil per week	Positive	8	27	27	25	4	26	27	24
(1) Ether soluble non-saponifiable matter of cottonseed oil = 2.5 gm. of the oil per week	Positive	4	16	18	12	8	18	21	13
(2) Acetone soluble extract of (1) = 11.2 gm. cottonseed oil per week	Positive	4	12	16	6	0	..	..	..
(3) Petroleum ether soluble material of (2) = 11.2 gm. of cottonseed oil per week	Positive	2	5	6	4	2	8	10	6
(4) Methyl alcohol soluble material of (3) = 11.2 gm. of cottonseed oil per week	Positive	2	2	3	1	6	3	4	1
Non-saponifiable matter of wheat germ oil treated with ferric chloride in ether = 2.5 gm. of wheat germ oil per week	Negative	2	1.5	3	0	2	2	3	1
Non-saponifiable matter of wheat germ oil heated to 120 C. = 2.5 gm. of wheat germ oil per week	Positive	2	— 1.5	1	— 3	2	— 11	— 14	— 8

With cottonseed oil as a starting point, numerous concentrates were made and fed to the test animal. The oil was subjected to a hot saponification (2 hours at 79°C.) and an ether extraction made, as in the case of the wheat germ oil. This extract, concentrate no. 1, when fed at an equivalent to 2.5 gm. of the original oil per week, showed both growth and anti-sterility effects. Subsequent purification of the vitamin was made as outlined by Olcott and Mattill ('31): The ether soluble material, concentrate no. 1 on table 1, was evaporated to dryness and dissolved in hot acetone. The acetone solution, concentrate no. 2, was cooled and filtered. The acetone was then evaporated and the residue extracted with petroleum ether. The petroleum ether solution, concentrate no. 3, was evaporated to dryness and extracted with methyl alcohol. The methyl alcohol soluble material was concentrate no. 4. The entire series of operations was carried out under carbon dioxide, using freshly distilled solvents. As can be seen from a consideration of table 1, the growth effect diminished as the purification of the vitamin proceeded, concentrate no. 4 showing practically no growth-stimulating potency and yet containing ample amount of vitamin E for the anti-sterility effect. The supplements, vitamin E concentrates, were administered with a small portion of the ration in the evening. By morning this had been consumed and ample diet for the day was placed in the feed cups. Concentrates nos. 2, 3 and 4 were fed at a level equivalent to 11.2 gm. of the original oil per week, this amount being divided equally into seven daily portions.

An attempt was made to destroy the vitamin E by treatment with ferric chloride (Waddell and Steenbock, '28) without altering the growth-promoting power of a wheat germ oil concentrate. The results showed a failure of the treated extract both in growth stimulation and in anti-sterility effect when fed at a level equal to 2.5 gm. of wheat germ oil per week.

The heating of the non-saponifiable matter of wheat germ oil to 120°C. for 1 hour in an autoclave, which should not

destroy vitamin E (Sure, '26), resulted in destruction of the factor in the concentrate which stimulated growth. The presence of sufficient vitamin E for anti-sterility effect was demonstrated in the two females treated with this preparation at a level equal to 2.5 gm. of wheat germ oil per week.

DISCUSSION

There are two possible explanations of the results found. First, a great difference exists between the amount of vitamin E required for anti-sterility effect and for growth; second, the concentrate formerly considered to contain one vitamin actually contains at least two—one growth stimulating and one having an anti-sterility effect. The evidence presented favors the possibility of a multiplicity of factors. Two preparations, the concentrate no. 4 and the autoclaved wheat germ oil concentrate, showed no growth effect and yet showed an anti-sterility potency in females of proved sterility. The preparation of crystalline α-tocopheryl allophanate, from which pure vitamin E was regenerated by Evans and coworkers ('36), should permit definite proof of this point when the preparation is available in sufficient amounts.

Suggestions have previously been made that vitamin E is in reality a complex. Grijns and Dingemanse ('33) obtained an extract of wheat germ which cured sterility in the male but not in the female. A second concentrate cured the female sterility and not the male. These authors used 40% alcohol in the presence of calcium hydroxide and kieselguhr for their separation. Blumberg ('35) suggested a possible growth and anti-sterility complex in wheat germ oil concentrates. Recently, Evans et al. ('36) by means of silver nitrate reduction tests, demonstrated that vitamin E activity is not the property of a single molecular species. This opens up the possibility that the two effects, growth and anti-sterility, may be due to slight alterations in a basic structure.

CONCLUSIONS

A report is made of an attempt to separate vitamin E concentrates into two fractions, one effective in curing sterility in rats and one effective in stimulating growth in these animals. The results show the presence of a factor stimulating growth, in concentrates formerly considered to exert only an anti-sterility effect.

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ADSORPTION OF VITAMIN B BY PLANT TISSUE (BY SOLANUM MELONGENA LINN. AND RAPHANUS SATIVUS VAR. LONGIPINNATUS BAILEY) WHEN PICKLED WITH SALT AND RICE BRAN [1]

CAREY D. MILLER

Nutrition Laboratory, Hawaii Agricultural Experiment Station, Honolulu [2]

ONE FIGURE

In a previous report (Miller and Abel, '33), it was shown that when a leafy vegetable, Chinese cabbage, Brassica chinensis, is pickled with a paste of salt and rice bran, the vitamin B (B_1) content of the cabbage is increased to almost four times the original value. The hypothesis was that the vitamin B from the rice bran either penetrated the tissues of the cabbage or was held by adsorption on the surface of the leaves. This paper gives the results of similar experiments with two other types of plant tissue: a fruit used as a vegetable, long eggplant, Solanum melongena Linn., and a root vegetable, daikon, Raphanus sativus var. longipinnatus Bailey.

The long slender eggplant, ranging from 4 to 10 inches in length and from 1 to 2 inches in diameter, is preferred by the Japanese to the larger round or oval type. It is one of the vegetables which these people pickle at home, alone or with other vegetables, using salt and such accessory substances as rice water, sake residue, kogi (fermented rice), miso (fermented soy bean and kogi), and rice bran.

The long white radish or daikon is a much favored vegetable used in large quantities by the Japanese in Hawaii as in Japan. Pickled with salt and rice bran, it is called takuan,[3] and in this form or in the fresh state is utilized by the majority of Japanese families at every meal. Some takuan is prepared commercially in Hawaii, but most of it is imported from Japan. Small quantities are made at home by a few families.

<div style="text-align:center">METHODS</div>

For experimental purposes, a uniform method of pickling the eggplant was followed. The eggplants, 6 to 10 inches in length and $1\frac{1}{4}$ to $1\frac{1}{2}$ inches in diameter, were weighed. For each 100 gm. of vegetable, 119 gm. of rice bran, 21 gm. of table salt and 210 gm. of water were made into a paste. Only firm whole eggplants with unbroken skin and with stem and calyx in place were used. The fruits were rolled gently with the hand on a smooth surface to soften them slightly. They were then placed in alternate layers in a wooden shoyu tub with the paste of salt and rice bran. A wooden cover fitting loosely into the tub was placed on the contents and weighted with a stone. The eggplant and paste were mixed each day and left for 72 hours. The eggplants, washed free of bran and salt, were placed in a covered glass jar in an electric refrigerator until needed. To secure a uniform sample each day, a whole eggplant was chopped fine and the quantities fed to rats were weighed to within $\frac{1}{100}$ gm. A fresh lot of eggplant was bran-salt-pickled twice a week.

The product designated as takuan A was prepared in the nutrition laboratory as follows: Large daikon 12 to 15 inches long and $1\frac{1}{4}$ to 2 inches in diameter were washed gently so as not to break the skin, then cut crosswise into two approximately equal pieces. After the daikon were weighed, salt equal to 5% of their weight was sprinkled over the daikon placed in layers in a clean shoyu tub. A fitted board was placed on the daikon and weighted with a heavy stone. The

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[3] Takuan has been eaten in Japan for 300 years or more. The term Takuan (sometimes spelled takuwan) is usually associated with a priest of that name, who, if he did not originate the method of pickling, did much to popularize its use.

daikon were mixed once a day and were removed after 72 hours from the resulting brine. This brine was discarded and the pre-salted daikon weighed without washing. Salt equal to 4%, and rice bran equal to 50%, of the pre-salted weight of the daikon were mixed and placed in alternate layers with the daikon in the clean shoyu tub and weighted with wooden cover and stone as before. The daikon and bran paste were mixed once daily and allowed to pickle for 1 week. The pickled product, takuan, was taken out of the tub, washed thoroughly and stored in glass jars in an electric refrigerator. Because of the large size of each root, it was impossible to use a whole one each day for feeding purposes. Cross sections from the center of half a root were cut in small pie-shaped pieces and weighed to within $\frac{1}{100}$ gm. A fresh lot of takuan was prepared each week.

Takuan B, used for the experiments, was commercial takuan imported from Japan.

The feeding methods were those previously described (Miller and Abel, '33) with the additional use of the international standard vitamin B preparation for positive control rats. Daily feeding means every day except Sunday.

The pH values of the extracted plant juices, made by grinding the tissues and squeezing them in cheese cloth, were determined as previously reported (Miller and Abel, '33).

<center>RESULTS</center>

Eggplant. Forty-five rats given a vitamin B-free diet were used for the experiments and fed as listed in table 1. The results, summarized in figure 1 and table 1, show that rats receiving 2 gm. of fresh eggplant daily gained less than the rats fed the standard and that rats fed bran-salt-pickled eggplant equivalent to 2 gm. of fresh eggplant gained as much as those fed 3 gm. of the fresh vegetable.

The loss in weight resulting from pickling twenty-two samples of eggplant in salt-rice-bran-paste varied from 10 to 32% with an average of 17%. The quantity of bran-salt-pickled eggplant equivalent to 2 gm. of the fresh vegetable was calculated for each lot according to the loss in weight.

Samples of one eggplant from each of twelve lots of the vegetable purchased over a period of 4 months varied slightly in pH, from 5.1 to 5.7 with an average of pH 5.4. Twenty-two lots of bran-salt-pickled eggplant made from the twelve lots of fresh eggplant all showed a definite increase in acidity, ranging from pH 5.1 to 4.5 with an average of pH 4.8.

TABLE 1

Results of feeding fresh and bran-salt-pickled vegetables as the sole source of vitamin B

SOURCE OF VITAMIN B	NUMBER OF RATS	WEIGHT OF SUP-PLEMENT FED DAILY	AVERAGE WEIGHT OF RATS			AVERAGE GAIN IN 6 WEEKS	APPROXI-MATE UNITS PER 100 GM.
			Initial	When started on supple-ment	Final		
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Negative control	10	0.0	41	73[1]	53		
Standard	10	0.005	39	65	102	37	
Eggplant, fresh	6	2.0	38	69	94	25	20
Eggplant, fresh	7	3.0	42	74	130	56	
Eggplant, bran-salt-pickled	12	1.6–1.7[2]	40	72	131	59	40
Negative control	11	0.0	37	69[1]	54		
Standard	12	0.005	37	67	98	31	
Daikon, fresh	10	8.0	39	67	106	39	6
Takuan A (nutr. lab.)	11	0.5	38	65	113	48	110–120
Takuan B (commercial)	10	1.0	39	71	97	26	40

[1] Weight at the end of the depletion period.
[2] Equivalent to 2 gm. of fresh.

Daikon and takuan. Fifty-four rats given a vitamin B-free diet were used to test the vitamin B content of daikon and takuan. The results are summarized in table 1 and figure 1.

That fresh daikon contains little vitamin B is demonstrated by the fact that it was necessary to feed 8 gm. daily in order to secure a gain comparable to that of rats fed daily 0.005 gm. of the vitamin B standard.

Table 1 also shows that the fresh pickled daikon (takuan A) prepared in our laboratory had a greater vitamin B value than

the commercial product, 0.5 gm. of the freshly prepared takuan (takuan A) producing about double the gain in weight resulting from 1.0 gm. of the commercial takuan (takuan B). It is quite possible that the freshly made commercial preparation contains as much vitamin B as our laboratory preparation, but gradually decreases in vitamin B through standing.

Daikon loses much water as a result of the pickling process, the percentage loss being about the same after the pre-salting
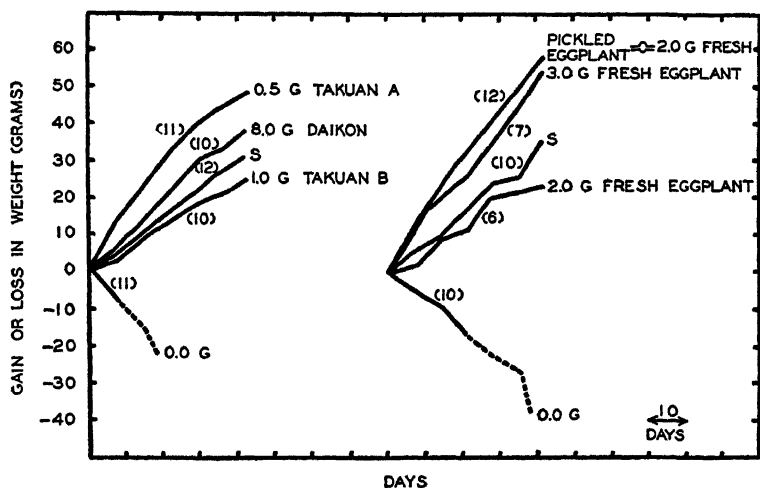
Fig. 1 Average gain in weight of rats fed fresh and bran-salt-pickled vegetables and the vitamin B standard. S = 0.005 gm. of the International Standard. The broken line indicates the occurrence of the first death in the control group. The numbers in parentheses show the number of rats used for each test.

as after the final pickling with salt and rice bran. The average loss in weight for nine samples was 64%, the minimum 62%, the maximum 74%. The weight of takuan equivalent to the fresh daikon was not calculated for each lot of takuan A prepared but 0.5 gm. was fed throughout the experiment.

The average pH of four samples of fresh daikon was 5.8, with little variation between the samples. As a result of the 3 days' pre-salting, there was practically no change in acidity, the average pH being 5.7. After a week in salt-rice-bran-paste, the average acidity increased to pH 4.8.

Limitations of space do not permit the inclusion of the results of preliminary experiments on twenty-five rats of which some were fed fresh daikon, some commercial takuan, and some used as negative controls. Eight of the group were matched litter mates fed equal portions of the outside and the inside of the takuan. When that portion representing one-third of the diameter of a cross section was taken from the center of the takuan root and fed to rats as the sole source of vitamin B, it induced just as great growth response as the outer portion. This shows that the vitamn B had penetrated to the very center of the root.

DISCUSSION

The extracted plant juices of the eggplant and of the takuan reached a pH of 4.8 as a result of the bran-salt-pickling process, the cabbage previously reported, a pH of 4.7 (Miller and Abel, '33). Acidity of about this pH has been shown to be most satisfactory for the adsorption of vitamin B on such substances as fuller's earth and Lloyd's reagent. The medium of rice bran, salt and moisture seems to create conditions favorable to the penetration and adsorption of vitamin B by any plant tissue.

A report from Japan (Kato and Ho, '30) states that the rice-bran paste from pickling vegetables contains many organisms of which a lactic acid bacillus is the most abundant. As in fermented cabbage (sauerkraut) and salt-cured cucumbers, lactic acid is the principal acid produced.

Dutcher ('32) and others have shown that certain micro-organisms possess the property of synthesizing the vitamin B complex. No study of the bacteria and yeasts involved in the bran-salt-pickling process has been made in the nutrition laboratory. However, since rice bran is extremely rich in vitamin B, a water soluble substance, it seems reasonable to assume that the vitamin B of the bran-salt-pickled products is derived from the original vitamin B of the bran; whether the quantity of vitamin B may or may not be augmented by micro-organisms present is a matter of little importance under these conditions.

Bran-salt-pickled eggplant contains per unit of weight only about twice the quantity of vitamin B as the fresh (table 1). Considering the loss in weight due to bran-salt-pickling, the increase in vitamin B content is only about $1\frac{1}{2}$ times the original value.

On the basis of units of vitamin B per 100 gm., takuan has about eighteen times as much vitamin B as the fresh daikon from which it is made (table 1). However, so much moisture is lost in the process of pickling that for the equivalent weight of fresh daikon, takuan contains only about seven times as much vitamin B as the original daikon.

Even though there is always considerable loss of water from the plant tissues as a result of the pickling process, nevertheless vitamin B, being water soluble, penetrates to the innermost tissues of the vegetables and is thus not removed by thorough, though not prolonged, washing in tap water. Our experiments show that, as a result of the bran-salt-pickling process, the vitamin B content of a leafy vegetable may be increased four times, of a fruit one and one-half times and of a root seven times its original value.

Due to its thick cuticle, the eggplant has a relatively impervious epidermis, a surface through which the vitamin B and salt can penetrate only slowly when the skin is unbroken and there are no cut ends. The daikon on the other hand has a more permeable epidermis and relatively less cuticle. Further, the ends were cut so that the soluble materials might easily penetrate the fibro-vascular bundles and, having once reached the innermost tissues, would not readily be lost.

In contrast with pickling in salt alone (Miller and Abel, '33), which decreases the original quantity of vitamin B, the general use of rice bran for pickling vegetables as a method of preservation in the orient and elsewhere is a practice much to be commended because it utilizes a product of high nutritive value (rice bran) otherwise little used for human consumption and because it results in an increased vitamin B intake where that vitamin is likely to be low.

SUMMARY

1. As a result of pickling long eggplant (Solanum melongena Linn.) in a paste of salt and rice bran for 3 days, the original vitamin B content of the eggplant is increased about one and one-half times.

2. Takuan, a product prepared from daikon (Raphanus sativus var. longipinnatus Bailey) by pickling in a paste of salt and rice bran for a week, has about seven times as much vitamin B as the original. Commercial takuan that had been pickled for some months had a vitamin B value less than half of that prepared in the laboratory.

3. As a result of pickling in a paste of salt and rice bran, plant tissues—whether leaf, fruit or root—reach a pH of 4.7 or 4.8, a condition that seems to be conducive to the adsorption and retention of vitamin B from the rice bran.

LITERATURE CITED

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SUPPLEMENT

PROCEEDINGS OF THE FOURTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

The fourth annual meeting of the American Institute of Nutrition was held in Memphis, Tenn., at the Hotel Peabody on April 21, 1937. Although only 90 members and 228 guests registered there were between 400 and 500 in attendance during the entire program.

President Eugene F. DuBois presided at the scientific sessions and business meeting. The scientific program was initiated promptly at 9.30 A.M. and with but few exceptions the speakers were thoughtful in consuming only the time allotted for the presentation of their papers. This helpful cooperation on the part of all the speakers enabled the entire program to move according to schedule and therefore with ease, satisfaction and enjoyment. All the papers listed on the program were given.

The business meeting convened at 11.30 A.M. and was called to order by Pres. Eugene F. DuBois.

The members stood in silence in recognition of the deaths of W. McKim Marriott and Winfred W. Braman.

The report of the nominating committee was presented by Arthur H. Smith, chairman. It was moved by Harold Goss and seconded by Paul E. Howe that the secretary cast a unanimous ballot for the slate as presented. The motion carried and the following officers were elected:

	Associate editors
President, Mary S. Rose	W. H. Chambers
Vice-president, E. V. McCollum	A. G. Hogan
Secretary, I. G. Macy	J. B. Brown
Treasurer, G. R. Cowgill	P. A. Shaffer
Councillor, P. E. Howe	J. L. Gamble

The report of the treasurer was read by George R. Cowgill. L. A. Maynard and C. A. Elvehjem, auditors reported the books correct. It was moved by Paul E. Howe and seconded by E. M. Nelson that the treasurer's report be accepted. The motion carried. The council recommends that the annual dues remain $1.00 during the coming year.

President DuBois appointed the following persons as members of the nominating committee for 1938: R. M. Bethke, chairman; A. H. Smith, W. M. Boothby, H. B. Lewis and Agnes F. Morgan.

The report of the committee on vitamin nomenclature was heard. E. M. Nelson, chairman of a committee composed of himself, E. V. McCollum and H. C. Sherman, after discussing the activities of the committee moved that the recommendations as submitted by the committee be approved. The motion was seconded by V. C. Myers and approved by the members. Although a large majority of the members favored dropping the final 'e' in the spelling of thiamine the final decision was left to the discretion of the committee. The committee was requested to continue its work.

The recommended changes in the By-Laws as submitted to the membership preceding the meeting in accordance with the constitution were discussed by President DuBois. He remarked that the council interprets Article VII to mean that the editor can be reappointed as stated in the by-laws. It was moved by R. M. Bethke and seconded by C. A. Elvehjem that the proposed changes be adopted. The motion carried.

President DuBois discussed a request from the Children's Bureau for qualifications of nutritionists in public health departments. In accordance therewith he appointed the following committee to study the request and make recommendations back to the council for their consideration: Mary S. Rose, chairman; R. M. Bethke, L. J. Roberts, W. C. Rose and Icie G. Macy.

The council recommended that a questionnaire be prepared and sent to the members to ascertain their wishes on the type of program desired and the place and time of meeting. A

committee composed of George R. Cowgill, R. M. Bethke and Icie G. Macy was appointed to draw up the questionnaire. President DuBois stressed the importance of suggestions from the members in guiding the council regarding nature of programs, etc. The council expressed the desire that the members of the institute themselves present the papers at the meeting since in their judgment it seems prudent at present at least to emphasize mature contributions rather than using the program for a training ground.

President DuBois announced that the council had charged R. M. Bethke and George R. Cowgill with the responsibility of submitting a resolution on the food, drug and cosmetic bill. The following statement was heard and approved by the members:

Resolved that the American Institute of Nutrition favors the passage of federal legislation which will control more effectively than at present the quality of and representations for food, drug and cosmetic products.

Mary S. Rose submitted the following resolution in connection with the work of the health organization of the League of Nations:

The American Institute of Nutrition endorses the report on Physiological Bases of Nutrition prepared by the Nutrition Committee of the Health Organization of the League of Nations as a guide for various countries for procuring optimum nutrition for their populations.

The recommendations regarding energy and protein requirements are in general harmony with standards approved by the members of the American Institute of Nutrition and the emphasis on liberal amounts of protective foods as sources of minerals and vitamins especialy for expectant and nursing mothers and for children of all ages is of the greatest significance for the welfare of the oncoming generation.

The report is commendable for calling attention to the importance of milk as a protective food and for setting standards in terms of common protective foods to which may be added such sources of energy as local conditions make practicable.

T. M. Carpenter moved that the resolution be adopted by the American Institute of Nutrition and sent to the medical director of the Health Organization of the League of Nations.

The council recommended that the 1938 meeting be held prior to the federation meeting.

It was moved by Paul E. Howe and seconded by E. M. Nelson that the secretary cast a ballot for the nine applicants recommended by the council for membership in the American Institute of Nutrition. The list follows:

H. A. Almquist	P. C. Jeans	G. Stearns
H. M. Dyer	J. M. Orten	J. Tilt
C. F. Huffman	W. H. Sebrell	A. White

John R. Murlin, managing editor, reported on The Journal of Nutrition. He stated that it would not be possible to increase from two to three volumes per year on account of finances. He expressed the hope that this might be possible in another year.

The council extended its thanks to the local committee and to the Hotel Peabody management for the splendid arrangements provided for the meeting and for the many courtesies extended to the members and their guests.

The meeting was adjourned.

Respectfully submitted,

ICIE G. MACY, *Secretary.*

CONSTITUTION

1. The name of the proposed society is the 'American Institute of Nutrition.'

2. The purposes of the society are to further the extension of the knowledge of nutrition and to facilitate personal contact between investigators in nutrition and closely related fields of interest.

3. The management of the American Institute of Nutrition shall be vested in a council consisting of the President, Vice-President, Secretary, Treasurer and three additional members.

Article I—*Membership*

Section 1. There shall be two classes of members, members and emeritus members. The number of members shall be limited to 250; exclusive of emeritus members.

Section 2. *Elegibility for membership*: Members.. Qualified investigators who have independently conducted and published meritorius original investigations in some phase of the chemistry or physiology of nutrition and who have shown a professional interest in nutrition for at least 5 years shall be eligible for membership in the Society. Emeritus Members. Members in good standing who have reached the age of 65 years shall become emeritus members. A member in good standing and for sufficient reason may by vote of the Council be made an emeritus member. Emeritus members shall be entitled to vote but not hold office.

Section 3. *Nomination*: Nominations for membership shall be made and seconded by members of the Society on blanks furnished by the Secretary. Nominations shall be submitted to the Council who shall determine eligibility and make recommendation to the Society at a regular meeting.

Section 4. *Election to membership*: A. A nominee for membership may be voted for by ballot at any meeting of the Society after the Council has reported its findings on his eligibility. B. A majority of the ballots cast shall elect.

Section 5. *Forfeiture*: If a majority of the Council after due notice to the member in question and opportunity for a hearing, shall decide that the interests of the Society require the expulsion of a member, the Secretary shall send a notice of this decision to each member at least 2 weeks before the next annual meeting. At this meeting the Secretary shall, on behalf of the Council, propose the expulsion; and if two-thirds of the members present vote for it, the member shall be expelled, his assessment for the current year shall be returned to him, and he shall cease to be a member of the Society.

Article II—*Meetings and Quorum*

Section 1. *Annual*: The annual meeting of the Society shall be held on the date fixed by the Certificate of Incorporation.

Section 2. *Special*: A special meeting may be called at any time by the President, or in case of his absence or disability, by the Vice-President, and must be called at the request in writing of a majority of the Council or fifteen members of the Society. Notice specifying the purpose of such meeting shall be mailed to each member at least 10 days previous thereto. The Council shall select the places at which meetings shall be held.

Section 3. *Quorum*: Fifteen members shall constitute a quorum at all meetings of the Society, but in absence of a quorum any number shall be sufficient to adjourn to a fixed date.

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[1] All amendments to April 21, 1937 are incorporated.

Article III—*Officials*

Section 1. *Officers*: The officers shall be a President, a Vice-President, a Secretary, and a Treasurer, who shall be elected annually by the members of the Society. Their terms of office shall commence with the close of the annual meeting at which they are elected.

Section 2. *Council*: The officers so selected and three additional members, one of whom shall be elected at each annual meeting to serve a term of three years, shall constitute a Board of Trustees and shall be known as 'The Council.' (When this provision is first put into effect one member shall be elected for 1 year, one for 2 years and the third for 3 years.)

Section 3. *Duties of Officers*: The powers and duties of the officers elected by the Society shall be such as usually devolve upon their respective positions.

Article IV—*The Council*

Section 1. *Powers*: The general management of the Society during the intervals between meetings shall be vested in the Council, which shall regularly perform the ordinary duties of an executive committee and possess all the powers conferred upon the Board of Trustees of an educational institution chartered by the Education Department of the University of the State of New York. A provisional charter was issued to the American Institute of Nutrition under date of September 27, 1928.

Section 2. *Reports*: The Council shall report to the Society its findings on the eligibility of candidates for membership, and on all charges of a violation of these By-Laws.

Article V—*Nominating Committee*

Section 1. *Membership*: A. The Nominating Committee shall consist of five members appointed for the coming year by the retiring President. Members who have served on the Nominating Committee for 2 consecutive years shall be ineligible for reappointment until after a lapse of 1 year. B. The President shall designate one member to be Chairman of the Nominating Committee.

Section 2. *Nomination of Officials*: A. The Nominating Committee shall make at least one nomination for each of the four offices, for each of the additional positions on the Council to be filled by vote of the members and for each of the positions on the Editorial Board to be vacated at the time of the annual meeting. Any member of the Institute may submit nominations to the Nominating Committee for its consideration along with those nominations made by the members of the Nominating Committee. B. The nominations by the Nominating Committee shall be transmitted to the Secretary at least 6 weeks before the annual meeting at which they are to be considered. C. The Secretary shall send to every member, at least 2 weeks before the annual meeting, a printed ballot containing the list of nominees and space for such additional names as the member wishes to propose, and at the same time shall notify the members that they may vote by mail, returning to the Secretary the marked ballot in the envelope provided, at such a time and place as the Secretary may designate, or the ballot may be delivered to the Secretary at the beginning of the business session at which the elections are to

take place. D. Additional nominations for the officers and for membership in the Council may be made by any member at the opening of the first executive session of any annual meeting.

Section 3. *Election of Officials*: A. At the beginning of the business session the Secretary shall present to the tellers, appointed by the President, the ballots submitted by the members and the ballots shall be counted forthwith. B. A majority of votes cast shall be necessary to elect an official.

Section 4. *Filling of Vacancies*: A. The Nominating Committee shall fill all vacancies in elective positions except such as may occur at a meeting of the Society. B. The President of the Society shall fill all vacancies in appointive positions.

Article VI—*Financial*

Section 1. *Dues*: The dues shall be the annual cost of subscription to The Journal of Nutrition for members plus an annual assessment which shall be determined by majority vote at the annual meetings, upon recommendation of the Council, and shall be due within a month after the annual meeting. Emeritus members are not required to pay assessments nor to subscribe to The Journal of Nutrition.

Section 2. *Expenditures*: No expenditures from the general funds of the Society except those required in the performance of the ordinary official duties shall be made except by vote of the Society or the Council.

Section 3. *Penalty for non-payment of dues*: A. Members in arrears for dues for 2 consecutive years shall forfeit their membership. B. Delinquent members may be reinstated by the Council provided all indebtedness to the Society is liquidated.

Article VII—*The Journal of Nutrition*

Section 1. The American Institute of Nutrition designates The Journal of Nutrition as its official organ of publication.

Section 2. In accordance with the expressed wish of The Wistar Institute of Anatomy and Biology, owner and publisher of The Journal of Nutrition, the American Institute of Nutrition shall nominate members of the Editorial Board for its official organ. A. The editorial management of The Journal of Nutrition shall be vested in an Editorial Board consisting of an Editor and twelve Associate Editors. B. The Editor shall be chosen by the Editorial Board to serve a term of 5 years. The Board shall also have the power to designate annually one or more of its members to serve as Assistant to the Editor. C. Three members of the Institute shall be nominated by the Nominating Committee for membership on the Editorial Board each year to serve a term of 4 years, replacing three retiring members. In the event of a vacancy in the membership of the Editorial Board occurring through death or other reason, the Nominating Committee, for each such vacancy to be filled shall make an additional nomination. In this event the nominees elected who receive the greatest number of votes shall serve the longest term of vacancies to be filled. D. Retiring members of the Editorial Board shall not be eligible for renomination until 1 year after their retirement.

<center>Article VIII—*Papers on Scientific Subjects*</center>

Section 1. The Secretary shall be authorized to arrange programs for the scientific sessions at the annual meetings.

<center>Article IX—*Changes in Constitution and By-Laws*</center>

Section 1. Proposed changes in the Constitution and By-Laws must be sent in writing to the Secretary at least 1 month before the date of the meeting at which they are to be considered, and must be signed by at least three members. The Secretary shall send a printed copy of any proposed change to each member at least 2 weeks before the next meeting and shall notify all members that they may vote by proxy.

Section 2. If at this meeting two-thirds of the votes cast shall favor the proposed change, it shall be made.

<center>ABSTRACTS OF PAPERS READ</center>

Organic vs. inorganic compounds as sources of mineral elements in nutrition. Genevieve Stearns (by invitation), Department of Pediatrics, State University of Iowa.

The retentions of sodium, potassium, calcium, phosphorus and iron have been studied when these elements were given to infants in foods, and as inorganic salts. In general either source was well utilized by the infant.

The quantity of intake of either of the monovalent ions studied was apparently without significant effect on the retention of any of the elements studied. The polyvalent ions, however, showed a greater degree of interrelationship. An oxalate containing food given to infants decreased the retentions of both calcium and iron.

The quantity of an element ingested, both in absolute amount, and in relation to the other minerals fed, seems to be of more importance for infant feeding than whether the element is fed as an organic or inorganic salt.

The influence of sex on utilization of iron. Helen J. Hubbell (by invitation) and Mary S. Rose, Nutrition Laboratory, Teachers College, Columbia University.

Rats made anemic by milk feeding were fed milk supplemented by iron and copper in amounts proportional to body weights until blood hemoglobin reached 14 gm. per 100 cc., when their bodies were analyzed for iron. Three levels of iron intake were studied.

A series fed 0.0019 mg. per gram of rat daily for a period averaging 45 days for seven males and 49 days for eight females, averaged for hemoglobin 14.3 gm. and 13.8 gm., respectively; for body iron 030.0 ± 0.0003 mg. per gram of rat for males and 0.038 ± 0.0007 mg. for females.

A series fed 0.038 mg. per gram of rat daily for a period averaging 39 days for eight males and 38 days for eight females, averaged for hemoglobin 14.1 gm. and 14.4 gm., respectively; for body iron 0.034 ± 0.0006 mg. per gram of rat for males and 0.038 ± 0.0007 mg. for females.

A series fed 0.0057 mg. of iron per gram of rat gave no evidence of further storage of iron but similar sex differences in iron content of bodies.

In each series the females showed a higher iron content by 13, 12 and 15%, respectively. No sex difference was found in the iron content of anemic controls analyzed at the end of the depletion period.

The nutritional status of children as judged by the creatinine-height coefficients. Amy L. Daniels, Iowa Child Welfare Research Station, State University of Iowa.

The study is concerned with the creatinine-height coefficients of pre-school children from two socio-economic groups. On the assumption that the creatinine-height is a measure of the nutritional status, these have been compared with the norms of weight for height, the predicted weights based on body build, and variations from the normal Pelidisi. In certain instances, the skeletal age has been considered in relation to these nutritional indices.

The calcium retentions of pre-school girls at different levels of calcium intake. Julia Outhouse, Gladys Kinsman (by invitation), Dorothy Sheldon (by invitation), Irene Twomey (by invitation), Janice Smith (by invitation) and Milicent Hathaway (by invitation), Department of Home Economics, University of Illinois, Urbana, and H. H. Mitchell, Department of Animal Husbandry, University of Illinois.

Five pre-school children were fed different levels of calcium in conjunction with a diet ample in essential nutrients. Excreta and food were collected and analyzed in weekly periods. During the first 15 weeks, generous amounts of di-calcium phosphate were fed, in order that saturation of the bones might be obtained. This procedure, it was assumed, would tend to erase differences in storage due to differences in the previous regimens of the children. During this period the retentions varied considerably from week to week and from child to child. Three children retained more calcium during the first 5 weeks than during the last 6 weeks. These results were reversed in the other children. At the end of 15 weeks, the quantity of calcium given was decreased to 350 mg. daily. This represented from two to seven times the amount of the average retention on the previous high level. Retentions were reduced in all but one child. At the end of the eighth week the calcium content was increased to 600 mg. daily through the addition of milk solids equivalent to 200 cc. milk. The increased retentions were proof that none of the children was receiving enough calcium on the preceding period of low calcium intake.

Effect of a milk supplement on bone development in institution children as indicated by ossification of bones of the wrist. Lydia J. Roberts and Vera MacNair (by invitation), Department of Home Economics, The University of Chicago.

The effect of adding a pint of milk, both irradiated and non-irradiated to the diets of institution children was studied over the period of a year. Roentgenograms of the wrist were taken at the beginning and end and the progress of bone development, as indicated by Carter ossification ratio, followed. Comparisons were made for twenty-four matched trios consisting of one child each from control, plain milk and irradiated milk groups.

All groups at the outset were retarded in respect to the Carter norms and were still retarded at the end of the study, but the supplemented groups reduced their deficits to a greater degree than did the control.

1. The percentage of children in more favorable relation to the standard at the end was 17 for the control, 50 for the milk, and 46 for the D-milk group.

2. The mean gain in Carter points was 6.0 for the control, 8.3 for milk and 8.2 for the D-milk group, an advantage of 2.3 and 2.2 points, approximately a third of a year's quota in favor of the supplemented groups.

3. The control group gained 79% of its expected quota; the supplemented groups each 115%.

4. In the twenty-four matched trios the control child made the greatest annual gain in 21%, the milk child in 44%, and the D-milk child in 35% of the comparisons.

Metabolism studies in human beings on the age disposition to ketosis. Walter Heymann (introduced by V. C. Myers), Babies and Children's Hospital, and the Department of Pediatrics, Western Reserve University.

Thirty-six persons of varying age (2 months to 35 years) were placed for 3 consecutive days on a ketogenic diet with a ratio of 2.5 : 1. Development of ketosis was detected daily by quantitative determination of acetone excretion in the urine. It was found that up to the age of 6 months ketonuria remains minimal. The ability to develop ketonuria begins at the age of 7 to 8 months, increases continuously during the first 3 years, reaches a peak at 4 to 8 years and decreases during prepuberty where it equals values obtained in adults, values which are still much higher than those found in young infants.

Differences in the production as well as in the utilization or excretion of ketone bodies may account for these results. It was found, however, that infants and children began to excrete acetone as well as sodium acetoacetate at the same level of dosage in a quantitatively comparable manner, when they were fed these compounds. Consequently, it must be concluded that the age disposition to ketosis is due to a difference in ability to produce acetone bodies.

Salt metabolism in muscular activity. D. B. Dill, Jost Michelsen (by invitation) and H. T. Edwards (by invitation), Fatigue Laboratory, Morgan Hall, Harvard University.

The need for increased intake of sodium chloride in periods of excessive sweat secretion is becoming generally recognized. The diets of athletes, of laborers in heavy industries, and of patients with fever should provide for extra-renal salt loss.

It is not generally known that salt concentration in sweat depends on acclimatization, environmental factors and personal idiosyncrasy. If work is undertaken in midwinter in an arid environment at 40 to 45°C., the concentration of salt in sweat may vary from 45 to 89 millimolar in different individuals. In one subject the concentrations were 87, 89 and 89, respectively, in three periods of work totaling 4½ hours. The water excretion was about 4 liters; only one-tenth as much was ingested and thirst was satisfied. Serum chloride decreased 4%, osmotic pressure increased 7%, and serum protein increased one-fourth. The urine became chloride-free, and heat cramps were experienced.

The same subject after acclimatization at Boulder City in the summer of 1932 walked for 7.1 hours in the desert with an air temperature of about 40°C. He excreted about 9 liters of water and drank two-thirds as much. Serum chloride remained constant and serum protein increased one-fifth. The indirect measurements at Boulder City indicated that the salt concentration in sweat was about one-third that found on the same subject in Boston without acclimatization to high temperature.

The influence of inorganic salts in the diet on the changes in electrolytes and water of the tissues of the rat. Ercel S. Eppright (by invitation) and Arthur H. Smith, Laboratory of Physiological Chemistry, Yale University.

Young, rapidly growing rats were fed a basal low-salt ration plus various mineral supplements in quantities normally consumed by rats eating ad libitum an adequate synthetic diet. Energy, protein, and vitamin intakes were restricted to the average consumption of rats on low-salt diet.

Analysis of tissues of animals sacrificed after 60 days showed that the dietary inorganic matter profoundly influences tissue hydration. Sodium chloride unsupplemented by other salts results in excessive hydration, general except for skeletal muscle. With potassium added to sodium chloride, the tendency toward excessive hydration is slightly reduced. When calcium is the predominant basic ion, dehydration prevails in many tissues.

With Osborne-Mendel salt mixture in the diet, there is close agreement between extracellular volumes of water as calculated from chloride or from sodium content of muscle and serum. Lacking all the salts or calcium and phosphorus, there is an excess of sodium in muscle unaccounted for by that in extracellular water as calculated from chlorides.

Potassium and sodium are mutually antagonistic to their retention in muscle; diminished potassium and augmented sodium accompany poor nutritional state. The ratio of potassium to sodium in muscle, much distorted on sodium chloride or sodium chloride plus potassium regimes, is normal when calcium is ingested.

The significance of dietary calcium in motivating shifts of electrolytes and water in tissues is emphasized by this study.

Vitamin B fractions. Their nomenclature and functions. C. A. Elvehjem, Department of Agricultural Chemistry, University of Wisconsin.

The designation and the function of six of the factors which have been included in the so-called B complex will be discussed according to the following outline:

1. Vitamin B or B_1. Antineuritic vitamin. Functions in carbohydrate metabolism, especially pyruvic acid metabolism. There is no evidence that it has any direct relationship to nerve degeneration.

2. Flavin. Lactoflavin. Designated vitamin G or B_2 by Kuhn, Karrer, Booher, Lepkovsky and Jukes, and others. Indispensability demonstrated for rats and chicks. Undoubtedly functions in human metabolism. Is the prosthetic group in flavo-proteins.

3. Antipellagric factor. Designated vitamin $B_2(G)$ by Elvehjem and Koehn human P-P factor or canine blacktongue factor by Birch, György, and Harris; 'filtrate factor' or 'factor 2' by Lepkovsky and Jukes. Cures pellagra-like symptoms in chicks and blacktongue in dogs. A concentrate of this factor has proved active in human pellagra.

4. Rat antidermatitis factor. Designated vitamin B_6 by György, by Chick and by Harris; 'Factor 1' by Lepkovsky and Jukes; and vitamin B by Hogan and Richardson. There is some variation in the reports on the stability of vitamin H and vitamin B_6. Function and relation to human nutrition not definite.

5. Antiparalytic factor. Designated vitamin B_4 by Reader and by Keenan et al. Necessary for normal brain structure. No work on relation to humans.

6. Factor W. Designated alcohol-ether precipitate factor by Elvehjem, Koehn and Oleson. Its function may be related to flavins and tissue respiration.

The tripartite nature of vitamin G. Samuel Lepkovsky and Myrtise E. Krause (by invitation), Poultry Division, University of California, Berkeley.

Vitamin G is now known to consist of three separate vitamins, one, lactoflavin, obtainable in crystalline form, and the other two available only as extracts. The latter two can be differentiated from each other biologically, one (factor 1)[1] curing a 'specific' dermatitis in rats. The other (factor 2) also cures a dermatitis in chicks. In rats it cures a disorder characterized by loss of hair sometimes accompanied by dermatitis, and a swelling of the eyelids of such severity they frequently stick together. Both are growth promoting in both rats and chicks. Factor 1 can be separated chemically from factor 2 by its adsorption on fullers' earth, and by its greater heat stability in alkaline medium.

An interrelationship apparently exists between factor 1 and both lactoflavin and factor 2. Rats maintained on minimal amounts of factor 1, will grow and remain free of dermatitis provided both lactoflavin and factor 2 are present in adequate amounts. They will, however, develop the 'specific' dermatitis and their growth will be disturbed if either lactoflavin or factor 2 is withheld or fed in inadequate amounts. Similar results were obtained by Birch and György[2] when linoleic acid was withheld. It is noteworthy that all three factors so physiologically interrelated with factor 1, will themselves cure some sort of skin disturbance.

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[1] Factor 1 is probably identical with vitamin B_6, the difference being that only vitamin B_1 and lactoflavin are fed for testing vitamin B_6, whereas vitamin B_1, lactoflavin, and factor 2 must be fed for factor 1 tests.

[2] Birch, T. W. and P. György 1935 Biol. Chem. J., vol. 30, p. 304.

Vitamin B_2 deficiencies as influenced by dietary carbohydrates. Agnes Fay Morgan, Bessie B. Cook (by invitation) and Helen G Davison (by invitation), Laboratory of Household Science, University of California.

Lactose, sucrose and cornstarch were used as 54 to 70% of the basal diets fed to young rats deprived of one or more of the vitamin B_2 factors. Growth, dermatitis, cataract formation, blood and urine sugar and calcium were determined on animals kept in individual screen-floored cages on these regimes for 8 to 16 weeks.

Three factors of the vitamin B_2 complex were used, a wheat germ preparation of vitamin B_6, a rice bran preparation of the filtrate factor and crystalline lactoflavin. Crystalline vitamin B_1 (Merck) was given in ample amount to all the rats. The three vitamin B_2 preparations were given each alone, all combinations of two together as well as all three together.

The lactose diet with no vitamin B_2 factors produced as good conditions as with B_6 and flavin. The filtrate factor, however, improved growth. The cornstarch diet was but little improved by addition of filtrate factor but much improved by both B_6 and flavin. The sucrose diet gave better growth than the others when supplemented by all the factors but apparently supplied none of them spontaneously.

Thus the intestinal flora supported by lactose produces flavin and B_6, cornstarch carries the filtrate factor or favors its intestinal formation, and sucrose neither carries nor allows the intestinal production of any of these factors.

The vitamin B requirement of children. The effect of varied ingestion of vitamin B upon the food consumption of children. Frederic W. Schultz and Elizabeth M. Knott (by invitation), Department of Pediatrics, The University of Chicago.

Quantitative studies over an extended period of time have been made of the total daily weights of food consumed by two groups of children. Group I included thirty-six ambulatory children, 5 to 10 years of age, living at the Country Home for Convalescent Crippled Children, which is affiliated with The University of Chicago. Group II included twenty-two girls between the ages of 9 and 11, whose health was excellent, and who had been living for some time under the optimum conditions afforded by the Mooseheart Home for Child Training. Following control periods, the vitamin B level of the diets, both for the crippled children and the normal children, was augmented first through use of a stabilized wheat germ product and second by means of crystalline vitamin B. When results were calculated on the basis of grams of food consumed per square meter of body surface, the increased intake of vitamin B increased the food consumption from 12.5% to 28.6%. The quantity of vitamin B in the diet which produced the highest food consumption was approximately 22 International standard units per kilogram of body weight for the younger children, and about 19 units per kilogram for the older children. This quantity of vitamin B is comparable to the amounts (reported in J. Nutrition, vol. 12 p. 597, 1936) which produced the highest retention of the vitamin. Twenty units of vitamin B per kilogram may therefore tentatively be considered as the optimum vitamin B requirement of children.

Panmyelophthisis with hemorrhagic manifestations in rats on a nutritional basis. Paul György (introduced by V. C. Myers), Babies and Children's Hospital and Department of Pediatrics, School of Medicine, Western Reserve University.

Rats kept on a vitamin B complex deficient diet supplemented with vitamin B_1 plus synthetic lactoflavin developed the characteristic picture of 'acrodynia' in 6 to 15 weeks. Treatment with potent purified B_6 concentrates, prepared from wheat germ or molasses, resulted in improvement of the specific dermatitis. This improvement, however, was followed in many cases not by complete recovery but

by severe anemia, together with symptoms of a hemorrhagic diathesis, such as blood effusions in the skin, epistaxis, melena, hematuria, and, frequently, bilateral adrenal hemorrhage. In all these cases the rat 'acrodynia' was in remission before the hemorrhagic diathesis and anemia became manifest. In several rats, spontaneous improvement of the specific 'acrodynia' was noticed without any change in the experimental conditions and without specific treatment as introduction to the hemorrhagic disease. Hemorrhagic manifestations and anemia were never observed in rats which had received Peters' eluate (acid-alcohol elution of a yeast extract from charcoal). In several cases, treatment in an early stage with Peters' eluate restored normal conditions.

Examination of the blood of rats in the acute stage of the disease revealed low hemoglobin percentage and low platelet and white blood cell count, with granulopenia. White blood cell counts of less than 1000, with no polymorphonuclear cells, were the rule.

The histological picture of the bone marrow was consistent with the diagnosis of panmyelophthisis, including aplastic anemia and aleukia hemorrhagica.

The state of ascorbic acid in plant tissues. E. J. Reedman (by invitation) and E. W. McHenry, School of Hygiene, University of Toronto.

Previous work in this laboratory has shown that many plant tissues contain vitamin C as free ascorbic acid, dehydroascorbic acid, and in a combined form which is not extracted by customary methods. Chemical estimations of vitamin C do not show the correct values unless all three forms are measured. Combined ascorbic acid is insoluble in trichloracetic and meta phosphoric acids. It can be hydrolyzed by heating or by an approriate acid treatment at room temperature. After hydrolysis the non-ascorbic acid part of the compound is precipitated by trichloracetic acid. This material is protein. After removal of the protein the solution behaves chemically exactly as if it were a solution of pure ascorbic acid. Increased titration values due to measurement of combined ascorbic acid are not attributable to destruction of ascorbic acid oxidase as has been stated by Mack and others. Ascorbic acid, liberated from the protein compound, is oxidized by the oxidase or by chemical means. Spectrophotometric analyses clearly indicate that the reducing substance in the protein compound is ascorbic acid. The characteristic absorption bands are removed by oxidation. Information is not available to indicate the physiological significance of the occurrence of this protein-ascorbic acid compound. Estimation of the amounts of combined ascorbic acid in a number of foods has been made.

The effect of the concentration of dietary egg white on the need for a protective factor given orally or parenterally. Helen T. Parsons, Jane G. Lease (by invitation) and Eunice Kelly (by invitation), Department of Home Economics, University of Wisconsin.

The pellagra-like nutritional disorder in the rat or chick, due to egg white, is characterized by a definite interrelationship between the egg white of the ration and a certain potent factor, i.e., factor X of Boas, not identical with any member of the vitamin B-complex so far clearly characterized.

This interrelationship is such that the greater the concentration of dietary egg white the greater the amount of the factor necessary to cure the nutritional disorder; this is demonstrable when the potent factor is given either orally or parenterally.

The implication of these data is that the interrelationship is metabolic in nature rather than due to changes in the ration mixture or in the digestive tract. Inasmuch as the syndrome has not been reported so far on purified rations containing no egg white, it has not yet been demonstrated that the protective factor is a vitamin or that it is required in normal nutrition.

Substituting egg white autoclaved for 6 hours at 15 pounds pressure for the raw egg white in the physiologically harmful rations leads to prompt cures; that this is due to a removal of the injurious qualities of the egg white rather than to a production of the potent factor by autoclaving is shown by the lack of benefit obtained by feeding autoclaved egg white if the raw egg white remains in the ration together with the autoclaved.

The lactic acid in human blood in relation to the respiratory quotient after the ingestion of hexoses. T. M. Carpenter, E. H. Bensley (by invitation), D. B. Dill and H. T. Edwards (by invitation), Nutrition Laboratory of the Carnegie Institution of Washington, Boston.

The lactic acid content of the venous blood of two human subjects was determined in experiments with 50 to 100 gm. of glucose, fructose, or galactose at 45-minute and 1-hour intervals for 4 hours after ingestion. The respiratory exchange was measured between the taking of the blood samples and the alveolar carbon dioxide followed at 5-minute intervals. Glucose caused a slight increase in the lactic acid for at least $1\frac{1}{2}$ hours. Galactose caused a fall of the alveolar carbon dioxide for the last 3 of the 4 hours and a very marked increase in lactic acid, but not the same amount with both subjects. The increase in lactic acid was the greatest after the ingestion of fructose and lasted for $2\frac{1}{4}$ to 3 hours. Corrections of the average respiratory quotients were made from the changes in the alveolar carbon dioxide and the lactic acid. The final results showed that the increases in the respiratory quotients after the ingestion of glucose and galactose were nearly the same but that the increases after fructose were definitely greater than the other two sugars. The end results of the metabolism of glucose and galactose are the same, but the metabolism of fructose is accompanied by either a more rapid combustion of carbohydrate or a greater formation of fat than with the other sugars.

Are endogenous urinary nitrogen and basal metabolism related in the growing rat? U. S. Ashworth (by invitation) and G. R. Cowgill, Laboratory of Physiological Chemistry, Yale University.

In order to test the recently formulated universal law that 2 mg. of endogenous urinary nitrogen are excreted for every calorie of basal heat produced 130 rats of various sizes were used. A measurement was made of the basal metabolism and endogenous nitrogen excretion of each individual.

Approximately linear trends were secured when the logarithms of either basal calories or endogenous nitrogen were plotted against the logarithms of body weight, the correlation coefficients being 0.95 and 0.94, respectively. When basal calories and endogenous nitrogen were correlated with each other the correlation coefficient was found to have a value of 0.87. However, the net correlation coefficient between these two variables was not significantly different from zero when the body weight effect had been eliminated by partial correlation. Indications are then, that the reported proportionality between the basal heat and endogenous nitrogen excretion of growing rats is only apparent and not real, and lies in the relationship of each of these to body weight.

The shape of the curve secured by plotting basal metabolism against endogenous nitrogen for growing rats is very similar to that of the ordinary growth curve. This suggests that during the period of most rapid growth the basal output of heat increases relatively faster than the basal output of nitrogen.

The influence of the percentage of protein upon the thermogenic (specific dynamic) effect and the net energy of the diet. T. S. Hamilton (introduced by H. H. Mitchell) Department of Animal Husbandry, College of Agriculture, University of Illinois.

In a series of twelve paired-feeding experiments, combined with carcass analyses of rats fed, it was found that the growth-promoting values of diets increased as the percentage of protein increased from 4 to about 18; remained constant between 18 and probably about 30%; and then decreased when 42% or more protein was present.

The thermogenic effect, being the total heat calculated to have been produced in excess of the basal, following the ingestion of a unit of food, was found to decrease as the percentage of protein increased from 4 to about 18; remain practically constant for diets containing between 18 and 30% protein; and then increase rapidly when 42% or more protein was present.

The net energy varied inversely with the thermogenic effect, being higher for diets containing between 18 and 30% protein, and lower for diets containing either less than 18 or more than 30% protein.

Percentages of protein varying from 4 to 54 affected neither the muscular activity of the rats nor the basal metabolism, although it was found that the basal metabolic rates of growing rats was readily influenced by the caloric level on which the animals had been subsisting. These data support the conception that the better balanced a diet is, in satisfying an animal's requirements, the smaller will be its thermogenic effect and the greater its net energy value.

The energy metabolism and mechanical efficiency of young boys. Clara M. Taylor (by invitation) and Grace MacLeod, The Nutrition Laboratory of Teachers College, Columbia University.

The energy expenditure of six healthy boys, 9 to 11 years of age, has been measured 1) under standard basal metabolism conditions, 2) while sitting quietly indulging in such activity as would involve only small finger muscles, and 3) while riding a bicycle ergometer of electric brake type.

Basal metabolism was measured by means of various forms of Benedict respiration apparatus. The average deviation of the results from Dreyer predictions was —10.1% (range, —17.7 to + 1.2%); from the Benedict-Talbot standards, + 4.3% (range, — 4.6 to + 14.3%); and from the Harris-Benedict predictions, + 4.6% (range, —2.7 to + 15.4%).

The activity studies were carried out in a respiration chamber patterened after that of Benedict and Ritzman. The Carpenter-Haldane gas analysis apparatus was used with it.

Total work performed in pedaling the bicycle ergometer was determined, as well as carbon dioxide production.

Average energy expenditure in forty-six quiet play periods was 2.47 Cals. per kilogram per hour. This is 53% above the average basal energy expenditure of 1.61 Cals. per kilogram per hour.

Average energy expenditure in thirty-five cycling periods was 5.03 Cals. per kilogram per hour, 212% in excess of the average basal energy expenditure.

Average gross efficiency in the thirty-five cycling periods was 15.8%. If net efficiency is calculated by deducting the basal metabolism it is found to be 23.1%; if calculated by deducting the sitting metabolism it becomes 31.5%.

A new automatic respiration calorimeter, and a study of the influence of sugars on heat production in the rat. W. M. Barrows, Jr. (by invitation), W. R. Murlin (by invitation), Pauline Nutter (by invitation) and John R. Murlin, Department of Vital Economics, University of Rochester.

An automatic respiration calorimeter for small animals has been constructed, which operates upon the following principle:

In addition to the heat produced by the animal, artificial heat is supplied to the interior of the calorimeter by means of an electric heater. The total heat supply to the calorimeter is automatically maintained constant by means of a self-balancing Wheatstone bridge circuit, which is actuated by resistance thermometers in the circulating water and which actuates a motor-driven rheostat. This rheostat is connected in series with the electric heater and with a recording watt meter.

In order to measure the rate of heat production by the animal, it is necessary only to know the (constant) rate of total heat supply, subtract from this the amount supplied electrically, as shown by the watt meter and correct for the latent heat of water vaporized.

The 'indirect heat' may be measured by either the open- or closed-circuit method.

The calorimeter was designed to measure the total heat produced simultaneously by twelve to eighteen rats over long periods.

Results were shown comparing such measurements on paired rats fed two equicaloric diets containing 68% glucose and sucrose, respectively. The heat production during several weeks was identical, expressed on a basis of surface area. Results also were shown comparing the effects of similar diets differing by a small known number of calories.

ABSTRACTS OF PAPERS READ BY TITLE

The chemical composition of the increment of growth at different rates of growth.
Marjorie Pickens (by invitation), William E. Anderson and Arthur H. Smith,
Laboratory of Physiological Chemistry, Yale University.

This study is an attempt to evaluate progressive changes in body composition
during growth under controlled dietary conditions. Four groups of male albino
rats of uniform initial age and weight were reared on diets which produced four
distinct levels of growth. These included rapid and 'normal' growth, and two
levels of retarded growth provided by gliadin-stunted animals and their calorie
controls. After the one hundred and tenth day both stunted groups were reali-
mented. The whole bodies of rats at birth, 14 days, and 21 days, and of animals
at three different ages from the four dietary groups were analyzed for water,
fat, protein, and ash. The average body composition of groups of eight rats
of each diet and age showed increase of fat and loss of water with age on all diets,
together with approximate constancy of protein content. The greatest variations
between individuals of the same group and between different groups were in fat
content. Fat, which was present to the extent of 24% in the bodies of the most
rapidly growing animals of 230 days of age, represented 40% of the weight
gained by these animals between the one hundred and tenth and two hundred and
thirtieth days. The proportion of fat in the gains during the period of limited
intake of energy was less than half of that in the gains of rapidly growing animals
of the same age, after realimentation it increased sharply.

The specificity of galactose as a cataract producing agent. Helen S. Mitchell,
Gladys M. Cook (by invitation), Oreana A. Merriam (by invitation), Home
Economics Research, Agricultural Experiment Station, Massachusetts State
College.

Galactose is now recognized as a unique causative agent in the experimental
production of lens opacities in rats. The effect of altering the type and amount
of other ingredients in the experimental ration has been investigated. Clinical
experience and chemical analyses of lenses have suggested other leads.

The cataract-producing action of lactose or galactose rations was not appreciably
altered by 1, type of supplementary carbohydrate (starch, dextrine, sucrose,
glucose); 2, type of protein (casein, dried meat, egg albumin, lactalbumin) or
adequate amounts (15 and 30%); 3, type of fat (Crisco, butter, mutton tallow,
cod liver oil) or amount (2, 11, 22 and 44%); 4, addition of excess cholesterol
(2½ and 5%); 5, amount of salt mixture (0, 4 and 10%); 6, excess calcium as
lactate (4, 6 and 8%); 7, shifting acid-base balance (4.76% Na citrate, 3.4%
NH_4Cl); 8, amount of water ingested; 9, vitamin C administered orally or injected;
10, deficiency or excess of vitamins B_1 and B_2; 11, ingestion of large doses of
dinitrophenol; 12, injection of lens antigen (from beef, sheep, guinea pig and rat).

Cataract producing action of galactose was hastened by lowering the protein
(5%) and slightly delayed by the addition of cystine (1, 2 and 3%). The protein
factor is being further investigated.

Nitrogen metabolism in premature infants: Comparative studies of human and cow's milk. H. H. Gordon (by invitation), M. A. Wheatley (by invitation) and E. Maples (by invitation), Children's Clinic, New York Hospital.

Balance studies of nitrogen and fat were made in six healthy premature male infants, ranging in age from 16 to 34 days and in weight from 1.45 to 2.72 kg. at the onset of observations. Urine and feces were collected separately in eighteen observations of 3 to 9 days (totalling 91 days) following fore periods of constant diet of 3 to 7 days.

Four infants received equivaloric diets of human and cow's milk mixtures containing approximately 125 calories and 2.75 gm. of protein per kilogram of body weight per 24 hours. The average retentions of nitrogen (dietary minus urinary and fecal nitrogen) for these short periods were 0.25 and 0.27 gm./kg./24 hours and the average percentages of utilization ($\frac{\text{nitrogen retained}}{\text{dietary minus fecal nitrogen}}$) 70.9 and 70.4% for human and cows' milk, respectively. These are considerably higher than the figures reported in full term infants under comparable conditions.

Increasing the protein (cows' milk) to 5 and 9 gm./kg./24 hours without increasing the calories increased the nitrogen retained to 0.36 and 0.45 gm./kg./24 hours, respectively, but the percentages of utilization dropped to 51 and 33%.

No defect in absorption of nitrogen was found. The absorption of fat from both human and cows' milk was less than the results reported for full term infants.

Malignant changes in forestomach mucosa of rats related to low protein (casein) diet and prevented with cystine. George R. Sharpless, Department of Laboratories, Henry Ford Hospital.

Hyperplasia of the squamous epithelium of the forestomach was observed in all of ninety-eight hooded and Wistar Institute albino strain rats fed a purified diet containing 4% casein and 5% yeast as sources of protein. This hyperplasia ranged from slight thickening and papilloma formation to extensive keratinization with the formation of epithelial cysts. The basement membrane is frequently broken through and the submucosa and muscle invaded by large hyperchromatic cells many of which are in mitosis.

The type of carbohydrate has no effect on the development of the lesion. Rats having a low food intake did not show as severe type of lesion as those which ate well (7 to 9 gm. daily). Neither vitamin A nor vitamin B deficiency increased the severity of the lesion, and an excess of either vitamin did not prevent it.

With 8% casein a less severe lesion developed after 4 months. Thirty-five rats fed 12% casein showed no lesions in first or second generations. Nineteen rats fed the basal diet with 0.2% cystine had no lesions. Ten milligrams of glutathione per rat daily or 0.2% cysteine hydrochloride did not prevent the lesion.

ROUND TABLE DISCUSSIONS

Total metabolism, insensible perspiration and skin temperature. L. H. Newburgh, presiding.

James D. Hardy opened the discussion on 'skin temperature' by describing his radiometric method of measuring skin temperature with absolute accuracy. The relation of skin temperature to peripheral circulation was discussed by Alan Burton and an index for measurement of this circulation in terms of skin temperature, body temperature and environmental temperature was presented. The application of the method of 'partitional calorimetry' to the measurement of the body heat lost by radiation, convection and evaporation, respectively, was described by A. P. Gagge. J. C. Hinsey concluded the discussion with a summation of the various autonomic nervous processes involved in the thermal responses of skin temperature to the environment.

L. H. Newburgh then introduced the subject of insensible loss of weight. He showed that the adult human being in a comfortable state loses close to 25% of his total heat by vaporization of water. One may therefore calculate total heat elimination from the weight of the water vapor. S. Z. Levine reported his studies on normal and premature infants. Max Kriss studying cattle in the respiration chamber found wide variations in this relationship. James M. Greene showed that this method of determining calorie output of rats, both in the basal and 24 hourly state, is satisfactory under carefully controlled conditions.

Nutrition in the clinic: The ideal liaison between the research worker and the clinician. Frederick F. Tisdall, presiding.

Seventy-four members and guests attended this discussion group. A free discussion ensued which showed the desirability of the clinicians being familiar with the rapid increase of scientific information relative to nutritional problems being obtained by the laboratory workers. On the other hand, the discussion served to give the laboratory workers a little insight into the difficulties which beset the clinicians. The closest possible cooperation between these two groups of workers was shown to be most desirable.

The discussion indicated the possible need for more of some of factors in the vitamin B-complex in the average daily diet; that there is great need for further clinical and laboratory work to be done on vitamin C in order to determine the daily human needs of this food element; recent laboratory work on vitamin D was reviewed and the fact that the human reacts to the different forms of vitamin D essentially in the same manner as the rat was emphasized, and the importance of the available iron and not the total iron in human nutrition was stressed.

At the conclusion of the meeting a vote was taken to find out whether the members present were in favor of discussion groups rather than a set address or series of papers. The vote was unanimously in favor of the discussion groups.

Methods of judging nutritional status. Lydia J. Roberts, presiding.

Lydia J. Roberts introduced the round table on the estimation of the nutritive status of children by reviewing the tests now in use and their deficiencies as well as by indicating the difficulties in devising objective physiological, chemical or

anthropometric estimations. Genevieve Stearns discussed the value of the biophotometer for measuring the adequacy of vitamin A intake and utilization and factors affecting its accuracy. Three thousand units of vitamin A had been found to be sufficient for boys 11 to 12 years. C. E. Palmer has not found the biophotometer exact in selecting children on repeated tests, but Genevieve Stearns explained that the method selected subjects with wider margins of adjustment rather than very fine.

Helen Hunscher described some of the possible benefits which might be derived from the use of estimations of skeletal maturity and degree of bone mineralization as judged by serial roentgen studies as means of selecting children for use in metabolic balance studies. Amy Daniels and Julia P. Outhouse contributed to this subject.

Gilbert Dalldorf opened the discussion on physiological means for measuring the adequacy of intake and utilization of vitamin C by human subjects. Estelle Hawley described the method for determining the daily requirement needed beyond that for saturation of body tissues by excretion studies. Fifty milligrams daily intake of ascorbic acid is suggested as a standard. R. A. Sloan reported on interrelated studies on rate of excretion, storage and level of vitamin C in the blood along with x-ray and capillary resistance tests. He indicated many factors which must be observed in these tests to secure reliable estimations. V. E. Levine described his experiences in measuring the vitamin deficiencies of the Eskimos.

It was concluded in this group discussion that there was a great need for adequate objective methods of evaluation of physical status as related to human nutrition.

Interconversion of foodstuffs. John R. Murlin, presiding.

Various aspects of the controversy over fat to carbohydrate occupied the larger part of the interconversion discussion under the leadership of J. R. Murlin. It was opened by H. J. Deuel, Jr. with an account of the transformation of amino acids, glycerol and odd-carbon fatty acids into carbohydrate as judged by liver glycogen analyses instead of the phlorhizin method. Data concerning a general correlation of the glycogen content of the body and the ability to oxidize carbohydrate were presented by W. H. Chambers. S. Soskin then spoke on the implications of considering the D: N ratio as derived from both fat and protein and the dominating position of the liver. The agreement between the chemical analyses of pupating insects and their low respiratory quotients at this time was shown by F. A. Hitchcock to support the fat to carbohydrate hypophysis. H. E. Himwick referred to the influence of methylene blue in lowering the R.Q. by the mechanism of the oxidation of hydrogen and then he broke up the quotient of the whole body into fractions contributed by the parts according to tissue determinations. This discussion led into the work of M. Elizabeth Marsh and the unseccessful attempts to explain quotients of 0.58 in liver slices by changes in acetone bodies, glycerol or protein metabolism. T. M. Carpenter pointed out the constancy of the height of the R.Q. after small or large doses of fructose, unaffected by muscular exercise, and emphasized the possible acid-base relationships. The discussion was closed on a less controversial note with a review by L. S. Palmer of the evidence for the conversion of carbohydrate to fat. About 150 people were in attendance, rather too many for intimate give and take in a group discussion.

Comparative nutrition—species differences as regards nutritive requirements and the effects of harmful food substances. L. A. Maynard, presiding.

After a few introductory remarks by L. A. Maynard, the following papers were presented:

1. Small animal studies as guides in human nutrition, by Arthur H. Smith, Department of Physiological Chemistry, Yale University.

2. Experiments with chicks in their relation to other species, by L. C. Norris, Department of Poultry Husbandry, Cornell University.

3. The nutritive requirements of the lower forms, by R. M. Melampy, Southern States Bee Culture Field Laboratory, Baton Rouge.

4. The vitamin requirements of guinea pigs and rabbits, by A. G. Hogan, Department of Agricultural Chemistry, University of Missouri.

Each paper was followed by rather vigorous discussion in which many of the sixty members present took part.

Expression of opinion from individuals indicated definite approval of this type of meeting.

Vitamin nomenclature. E. M. Nelson, presiding.

The meeting was called to order by the chairman whose opening remarks dealt with the scope of the problem. It was pointed out that there are more substances having the property of a vitamin than there are letters in the alphabet, and that there is obviously no hope for a system of nomenclature which will be so simple that the character of all the compounds can be readily comprehended. This was followed by the prearranged program in which W. C. Rose, Franklin C. Bing and A. D. Emmett partook. W. C. Rose stressed the inalienable right to a discoverer in naming a compound and also stated that anyone possessing such a right should give due recognition to systems of nomenclature and to problems which may arise in the use of names having wide application. F. C. Bing discussed the objections of the Council on Pharmacy and Chemistry to names which are therapeutically suggestive. A. D. Emmett emphasized the problems of the pharmaceutical manufacturer in teaching the physican and the layman the significance of names chosen for various compounds. Changes of names led to rather difficult situations.

In the discussion which followed particular attention was given to the desirability of giving specific names to vitamin A and to the various forms of vitamin D. There was no disposition on the part of the group to make definite recommendations with respect to these vitamins but the discussion will be very helpful to the committee on nomenclature. There was considerable discussion of the recommendations for vitamin B nomenclature which were approved at the business meeting of the institute. These recommendations met with general approval.

In discussing the objection of the Council on Pharmacy and Chemistry to names which are therapeutically suggestive, the chairman stated that he was cognizant of the fact that the council had been criticized in the past for its selection of names. He emphasized, however, that recognition must be given to the fact that there is a sound basis for objecting to names which are therapeutically suggestive. The council must, of course, interpret its own rules, and there is no need

for confusion if the position of the council is clearly understood and there is satisfactory cooperation between all interested groups.

The meeting was attended by approximately forty people of whom nearly one-half took part in the discussion. No one left the meeting before formal adjournment except those who had previously obtained permission to do so.

Data assembled by George R. Cowgill on the vitamin requirements of man. General meeting evening of April 21, 1937, Mary Swartz Rose, presiding.

Average American diet

	Vit./Cal.
Man (154 pounds) needs	1.97
Sherman's estimated averaged diet yields	2.74
121 family diets calculated separately averaged	2.77

Steffanson and Anderson ate an 'exclusive meat diet' for a period of 1 year and did not develop beriberi.

	Vit./Cal.
The diet yielded	2.77
Steffanson needed	1.96
Anderson needed	1.62

Sufficient vitamin B was indeed present.

Vitamin B and growth. Growth of rat requires from three to five times more than does maintenance (Dann). Children require for optimal retention of vitamin B from six to seven times the beriberi preventing minimum (Knott). Children in Toronto were not getting this amount (Ross and Summerfeldt).

Vitamin B for pregnancy and lactation. League of Nations Commission, 1935, recommends 150 to 250 International units per day, which is about 5 to 6 units per 100 cal. per day. This seems low. Safer minimum: at least 10 units per 100 cal. per day.

Vitamin B for the infant. Intake based on amount from human milk with maximum B content would be about 80 International units per day.

American Public Health Year Book, 1934–1935, recommends minimum of 50 units daily.

Vitamin A for pregnancy and lactation. League of Nations Commission, 1935, recommends: 9000 International units per day which is about 250 to 300 units per 100 cal. per day.

Vitamin A for the infant. Two thousand International units per day (Am. Public Health Year Book, 1934–1935). This amount means about 400 units per 100 cal. per day.

Good human milk contains 280 to 710 units per 100 cal. Cow's milk maximum is about 90 units per 100 cal.

Vitamin C in milk. Human milk maximum is about 1.4 to 1.6 International units per cubic centimeter. Thus the very young baby could get 1000 to 1200 units per day or 200 to 240 units per 100 cal. per day, or 2.75 units per kilogram per day. This is much more than most infants receive—cow's milk has much less, only 0.4 to 0.5 units per cubic centimeter.

Vitamin C for pregnancy and lactation. League of Nations Commission, 1935, recommends: 900 to 1100 International units per day or about 30 units per 100 cal. per day.

Vitamin D for the infant. To prevent rickets: 700 International units per day (Am. Public Health Year Book, 1934–1935) or 140 units per 100 cal. per day or 200 units per kilogram per day.

PAST MEETINGS, OFFICERS AND DECEASED MEMBERS

Organization meeting—1933—Henry C. Sherman, Chairman

1934 New York city

President: L. B. Mendel
Vice-President: H. C. Sherman
Secretary-Treasurer: J. R. Murlin
Council: E. F. DuBois, M. S. Rose

1935 Detroit

President: J. R. Murlin
Vice-President: E. F. DuBois
Secretary: I. G. Macy
Treasurer: W. M. Boothby
Council: A. F. Morgan, A. H. Smith, R. M. Bethke

1936 Washington

President: J. R. Murlin
Vice-President: E. F. DuBois
Secretary: I. G. Macy
Treasurer: G. R. Cowgill
Council: R. M. Bethke, L. A. Maynard, A. H. Smith

1937 Memphis

President: E. F. DuBois
Vice-President: M. S. Rose
Secretary: I. G. Macy
Treasurer: G. R. Cowgill
Council: R. M. Bethke, L. A. Maynard, C. A. Elvehjem

Deceased members of American Institute of Nutrition

GRAHAM LUSK, July 18, 1932
ALFRED F. HESS, December 5, 1933
A. W. ROWE, December 6, 1934
LAFAYETTE B. MENDEL, December 9, 1935
WINFRED W. BRAMAN, March 24, 1937
W. McKIM MARRIOTT, November 11, 1936

LIST OF MEMBERS

ADOLPH, WILLIAM HENRY, A.B., Ph.D., Professor of Biochemistry, Yenching University, Peiping, China.

ALMQUIST, H. A., Ph.D., Assistant Professor Poultry Husbandry, Division of Poultry Husbandry, University of California, Berkeley, Calif.

ANDERSON, WILLIAM E., M.A., Laboratory of Physiological Chemistry, Sterling Hall of Medicine, Yale University, New Haven, Conn.

ASCHAM, LEAH, Ph.D., Director Home Economics Research, Georgia Experiment Station, Experiment, Ga.

AUB, JOSEPH C., A.B., M.D., Associate Professor of Medicine, Harvard Medical School, Collis P. Huntington Memorial Hospital, Boston, Mass.

AXTMAYER, JOSEPH H., B.S., A.M., Ph.D., School of Tropical Medicine, Associate Professor of Chemistry, San Juan, Puerto Rico.

BAILEY, CAMERON V., M.D., C.W., Professor of Clinical Medicine, New York Post Graduate Medical School, Columbia University, 303 E. 20th St., New York, N. Y.

BATCHELDER, ESTHER L., B.S., A.M., Ph.D., 3 Riverview Road, Rocky Hill, Conn.

BEARD, HOWARD H., B.S., M.A., Ph.D., Professor of Biochemistry, Louisiana State University, Medical Center, 1542 Tulane Ave., New Orleans, La.

BENEDICT, FRANCIS G., Ph.D., Sc.D., Director of Nutrition Laboratory, Carnegie Institution of Washington, 29 Vila St., Boston, Mass. Member National Academy of Sciences.

BERG, CLARENCE P., A.B., M.A., Ph.D., Assistant Professor of Biochemistry, Chemistry Dept., University of Iowa, Iowa City, Iowa.

BERGEIM, OLAF, M.S., Ph.D., Associate Professor of Physiological Chemistry, University of Illinois, College of Medicine, 1817 W. Polk St., Chicago, Ill.

BETHKE, ROLAND M., B.S., M.S., Ph.D., In charge of Nutritional Investigations, Ohio Agricultural Experiment Station, Wooster, Ohio.

BILLS, CHARLES E., B.S., M.A., Ph.D., Research Director, Mead Johnson & Co., Evansville, Ind.

BING, FRANKLIN C., A.B., Ph.D., Secretary, Council on Foods, American Medical Association, 535 N. Dearborn Ave., Chicago, Ill.

BISBEY, BERTHA, B.S., A.M., Ph.D., Associate Professor of Home Economics, Gwynn Hall, University of Missouri, Columbia, Mo.

BISCHOFF, FRITZ E., B.S., M.S., Ph.D., Chief Chemist, Chairman of Research Com. Cottage Hospital, Santa Barbara, Calif.

BLACKFAN, KENNETH D., M.D., Professor of Pediatrics, Harvard University, 300 Longwood Ave., Boston, Mass.

BLATHERWICK, NORMAN R., M.S., Ph.D., Sc.D., Director, Biochemical Laboratory, Metropolitan Life Insurance Co., 1 Madison Ave., New York, N. Y.

BLOCK, RICHARD JOSEPH, B.S., Ph.D., Research Assistant in Chemistry, N. Y. State Psychiatric Institute, 722 W. 168th St., New York, N. Y. Residence, 15 Cooper Rd., Scarsdale, N. Y.

BOOHER, LELA E., M.S., Ph.D., Instructor in Chemistry, Columbia University, Box 26, New York, N. Y.

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BOOTHBY, WALTER M., A.B., M.D., A.M., F.A.C.S., Chief of Section of Clinical Metabolism in Division of Medicine, Mayo Clinic; Associate Professor of Medicine, Mayo Foundation, University of Minnesota; Metabolism Laboratory, Mayo Clinic, Rochester, Minn.

BOSWORTH, ALFRED, M.D., Honorary Fellow Physiological Chemistry, Ohio State University, 381 W. 10th Ave., Columbus, Ohio.

BRODY, SAMUEL, B.A., M.A., Ph.D., Associate Professor, College of Agriculture and Agricultural Experiment Station, Dairy Bldg., University of Missouri, Columbia, Mo.

BROOKES, MARGARET C. HESSLER, A.B., A.M., Ph.D., Assistant Professor, Dept. Home Economics, The University of Chicago, Chicago, Ill.

BROWN, JOHN B., B.S., M.S., Ph.D., Associate Professor of Physiological Chemistry, College of Medicine, Ohio State University, Columbus, Ohio.

BRUGER, MAURICE, B.Sc., M.D., C.M., M.Sc., Assistant Professor of Clinical Medicine, New York Post-Graduate Medical School, Columbia University, 245 E. 17th St., New York, N. Y.

BULGER, HAROLD A., Ph.C., B.S., M.D., Assistant Professor of Medicine, Washington University, St. Louis, Mo., care of Barnes Hospital, 600 S. Kingshighway, St. Louis, Mo.

BURR, GEORGE O., A.B., M.A., Ph.D., Associate Professor of Botany, Dept. Botany, University of Minnesota, Minneapolis, Minn.

BUTTS, JOSEPH S., M.S., B.S., Ph.D., Assistant Professor of Biochemistry, University of Southern California Medical School, Los Angeles, Calif.

CAJORI, FLORIAN A., Ph.D., Assistant Professor Physiological Chemistry, University of Pennsylvania Medical School, Philadelphia, Pa.

CALDWELL, MARY L., A.M., Ph.D., Assistant Professor of Chemistry, Dept. Chemistry, Havemeyer Hall, Columbia University, New York, N. Y.

CAMPBELL, H. LOUISE, B.A., Ph.D., Research Assistant in Food Chemistry, Columbia University, 435 W. 119th St., Apt. 9-F, New York, N. Y.

CARLSON, A. J., A.M., Ph.D., M.D., LL.D., Professor of Physiology, The University of Chicago, Hull Physiological Laboratory, Chicago, Ill. Member National Academy of Sciences.

CARPENTER, THORNE M., B.S., Ph.D., Physiological Chemist, Nutrition Laboratory of the Carnegie Institution of Washington, 29 Vila St., Boston, Mass.

CATHCART, E. P., M.D., D.Sc., LL.D., Dean, University of Glasgow, Scotland.

CHAMBERS, WILLIAM H., B.S., M.D., Ph.D., Assistant Professor Dept. Physiology, Cornell University Medical College, 1300 York Ave., New York, N. Y.

CHITTENDEN, RUSSELL H., Ph.D., LL.D., Sc.D., Professor Physiological Chemistry (Emeritus), Yale University; Member National Academy of Sciences, 83 Trumbull St., New Haven, Conn.

COOLEY, THOMAS B., A.B., M.S., M.D., Chairman of Staff, Children's Hospital of Michigan, Detroit, Mich. 1728 Seminole Ave., Detroit, Mich.

COONS, CALLIE MAE, B.A., B.S., Ph.D., Associate Food Economist, Bureau of Home Economics, Washington, D. C.

COWGILL, GEORGE R., B.A., Ph.D., Associate Professor of Physiological Chemistry, Yale University, 333 Cedar St., New Haven, Conn.

COX, GERALD J., B.S., M.S., Ph.D., Senior Industrial Fellow, Mellon Institute of Industrial Research, Pittsburgh, Pa.

CULLEN, GLENN E., A.B., B.Ch.E., Ph.D., Director of Laboratories, Professor of Biochemistry, Graduate School, University of Cincinnati; Prof. of Research Pediatrics, School of Medicine, University of Cincinnati, Children's Hospital, Research Foundation. Cincinnati, Ohio.

DAGGS, RAY G., B.S., Ph.D., Associate Professor of Physiology, School of Medicine, University of Vermont, Burlington, Vt.

DANIELS, AMY L., B.S., Ph.D., Research Professor of Nutrition, State University of Iowa, 10 E. Market St., Iowa City, Iowa.

DAY, PAUL L., A.B., M.S., Ph.D., Professor of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock, Ark.

DEUEL, HARRY J., JR., B.A., Ph.D., Professor of Biochemistry, University of Southern California Medical School, 3551 University Ave., Los Angeles, Calif.

DILL, DAVID BRUCE, B.S., A.M., Ph.D., Associate Professor Industrial Physiology, Fatigue Laboratory, Soldiers' Field Station, Harvard University, Boston, Mass.

DRABKIN, DAVID L., A.B., M.D., Assistant Professor of Physiological Chemistry, Dept. Physiological Chemistry, Medical School, University of Pennsylvania, Philadelphia, Pa.

DRAKE, T. G. H., M.B., F.R.C.P.(c), Junior Demonstrator in Paediatrics, Dept. of Medicine, University of Toronto, and Clinical Assistant on Active Staff and Associate Director of Research Laboratory, Hospital for Sick Children, Toronto, Ont.

DuBOIS, EUGENE F., A.B., M.D., Professor of Medicine, Cornell University Medical College; Medical Director Russell Sage Institute of Pathology, New York Hospital, 525 E. 68th St., Residence, 1215 Park Ave., New York, N. Y.. Member National Academy of Sciences.

DUTCHER, R. ADAMS, B.S., M.S., M.A., D.Sc., Professor and Head Dept. Agriculture and Biochemical Chemistry, Pennsylvania State College, 254 East Hamilton Ave., State College, Pa.

DYE, MARIE, B.S., M.S., Ph.D., Dean, Division Home Economics; Prof. of Nutrition, Michigan State College, East Lansing, Mich.

DYER, HELEN M., A.B., M.S., Ph.D., Associate in Biochemistry, George Washington University, Washington, D. C.

EDDY, WALTER H., B.S., A.M., Ph.D., Professor of Physiological Chemistry, Teachers College, Columbia University, New York, N. Y.

ELIOT, MARTHA M., A.B., M.D., Assistant Chief, U.S. Children Bureau, Washington, D. C.

ELLIS, N. R., B.S., M.S., Biochemist, Nutrition Laboratory, U. S. Animal and Husbandry Experiment Farm, Beltsville, Md.

ELVEHJEM, CONRAD A., M.S., Ph.D., Professor of Agricultural Chemistry, Agricultural Chemistry Bldg., University of Wisconsin, Madison, Wisc.

EMMETT, ARTHUR D., B.S., M.A., Ph.D., Nutritional Chemist, Research Laboratories, Parke Davis & Co., Detroit, Mich.

FINE, M. S., Ph.B., Ph.D., Director of Research, General Foods Corp., Battle Creek, Mich.

FITZ, REGINALD, A.B., M.D., Associate Professor of Medicine, Harvard Medical School, 721 Huntington Ave., Boston, Mass.

FORBES, ERNEST BROWNING, B.S., Ph.D., Director, Institute of Animal Nutrition, Pennsylvania State College, 500 Fairmount Ave., State College, Pa.

GAMBLE, JAMES L., A.B., M.D., S.M. (hon), Associate Professor of Pediatrics, Harvard Medical School, 33 Edge Hill Rd., Brookline, Mass.

GEYELIN, H. RAWLE, A.B., M.D., Assistant Professor of Clinical Medicine, College of Physicians and Surgeons, Columbia University; Attending Associate Physician, Presbyterian Hospital, 103 E. 78th St., New York, N. Y.

GIBSON, ROBERT BANKS, B.Ph., Ph.D., Associate Professor of Biochemistry, State University of Iowa, Iowa City, Iowa.

GOSS, HAROLD, B.S., Ph.D., Associate Professor of Animal Husbandry, University of California, Davis, Calif.

GREENWALD, ISIDOR, A.B., Ph.D., Associate Professor of Chemistry, New York University College of Medicine and Bellevue Hospital, 477 First Ave., New York, N. Y.

GRIFFITH, FRED R., JR., M.A., Ph.D., Associate Professor of Physiology, University of Buffalo Medical School, 24 High St., Buffalo, N. Y.

GRIFFITH, WENDELL H., M.S., Ph.D., Associate Professor of Biological Chemistry, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, Mo.

GUERRANT, N. B., B.A., M.S., M.D., Associate Professor Biological Chemistry, Pennsylvania State College, State College, Pa.

GULICK, ADDISON, A.M., Ph.D., Professor of Physiological Chemistry, University of Missouri, 308 Westmount Ave., Columbia, Mo.

HALLIDAY, NELLIE, B.S., A.M., Ph.D., Department of Zoölogy, University of Rochester, Rochester, N. Y.

HART, EDWIN B., B.S., Professor of Agricultural Chemistry, University of Wisconsin, Madison, Wisc.

HAUGE, SIGFRED M., B.A., M.S., Ph.D., Research Associate in Biochemistry, Purdue University Agricultural Experiment Station, Lafayette, Ind.

HAWLEY, ESTELLE E., B.S., M.S., Ph.D., Research Fellow in Pediatrics, University of Rochester Medical School, Rochester, N. Y.

HELLER, V. G., B.S., Ph.D., Professor and Head Dept. Agricultural Chemistry Research, Oklahoma A. and M. College, Stillwater, Okla.

HIGGINS, HAROLD L., A.B., M.D., Assistant Professor of Pediatrics, Harvard University, Massachusetts General Hospital, Boston, Mass.

HIMWICH, HAROLD E., B.S., M.D., Professor of Pharmacology and Physiology, Albany Medical College, Albany, N. Y.

HITCHCOCK, FRED A., M.Sc., Ph.D., Associate Professor of Physiology, Ohio State University, Columbus, Ohio.

HOGAN, ALBERT G., B.S., A.M., Ph.D., Professor of Animal Nutrition, University of Missouri, 105 Schweitzer Hall, Columbia, Mo.

HOLMES, ARTHUR DUNHAM, B.S., Ph.D., Director of Research, The E. L. Patch Co., 38 Montvale Ave., Stoneham, Mass.

HOPPERT, C. A., B.A., M.S., Ph.D., Professor of Biological Chemistry, Michigan State College, Box 626, East Lansing, Mich.

HOWE, PAUL E., B.S., M.A., Ph.D., Chief, Animal Nutrition Division and Assistant Chief Bureau of Animal Industry, U. S. Dept. of Agriculture, Washington, D. C.

HOWE, PERCY R., M.D., D.D.S., Director Forsyth Dental Infirmary; Professor of Dental Science; Instructor Pathology, Harvard Medical School, Boston, Mass.

HUBBELL, REBECCA B., A.B., M.S., Ph.D., Assistant Biochemist, Connecticut Agricultural Experiment Station, New Haven, Conn.

HUFFMAN, C. F., B.S., M.S., Ph.D., Research Associate and Associate Professor in Dairy Husbandry, Michigan State College, East Lansing, Mich.

HUNSCHER, HELEN A., A.B., Ph.D., Associate in Research, Children's Fund of Michigan, 660 Frederick St., Detroit, Mich.

HUNTER, JESSE E., B.S., M.S., Ph.D., Associate Professor, in Charge of Section of Poultry Nutrition, Pennsylvania State College, State College, Pa.

IVY, ANDREW CONWAY, M.S., Ph.D., M.D., Nathan Smith Davis Professor of Physiology and Professor of Pharmacology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, Ill.

JACKSON, RICHARD W., Ph.D., Assistant Professor of Biochemistry, Cornell University Medical School, 1300 York Ave., New York, N. Y.

JEANS, PHILIP C., A.M., M.D., Professor of Pediatrics, State University of Iowa, Iowa City, Iowa.

JONES, DAVID BREEZE, A.B., Ph.D., Principal Chemist in Charge Protein and Nutrition Division, Bureau of Chemistry and Soils, U. S. Dept. of Agriculture, Washington, D. C.

JONES, JAMES HAZLETT, B.S., M.S., Ph.D., Assistant Professor of Physiological Chemistry, University of Pennsylvania School of Medicine, Philadelphia, Pa.

JOSLIN, ELLIOTT PROCTOR, A.B., M.A., M.D., Director, George F. Backer Clinic, New England Deaconess Hospital, 81 Bay State Rd., Boston, Mass.

KEILES, ELSA ORENT, B.S., D.Sc., Associate in Biochemistry, School of Hygiene, Johns Hopkins University, 615 N. Wolfe St., Baltimore, Md.

KENNEDY, CORNELIA, B.A., M.S., Ph.D., Associate Professor of Agricultural Biochemistry, University Farm, St. Paul, Minn.

KING, CHARLES GLENN, B.S., M.S., Ph.D., Professor of Chemistry, University of Pittsburgh, Pittsburgh, Pa.

KLEIBER, M., D.Sci. Privat Docent, Associate Animal Husbandman, College Park, Davis, Calif.

KLEINER, ISRAEL S., Ph.B., Ph.D., Professor of Physiological Chemistry, New York Homeopathic Medical College and Flower Hospital, 64th St. and York Ave., New York City.

KLETZIEN, SEYMOUR W., B.S., M.S., Ph.D., Biochemist, State Institute for the Study of Malignant Disease, 113 High St., Buffalo, N. Y.

KLINE, O. L., Ph.D., Associate Chemist, U. S. Dept. Agriculture, Food and Drug Administration, Washington, D. C.

KNUDSON, ARTHUR, A.B., Ph.D., Professor of Biochemistry, Albany Medical College, Medical Department of Union University, Albany, N. Y.

KOCH, FRED CONRAD, M.S., Ph.D., Professor of Physiological Chemistry, Chairman of Dept. of Physiological Chemistry and Pharmacology, The University of Chicago, 951 E. 58th St., Chicago, Ill.

KOEHNE, MARTHA, B.A., M.A., Ph.D., Nutritionist, Ohio State Department of Health, Columbus, Ohio.

KRAMER, MARTHA M., B.S., Ph.D., Professor, Food Economics and Nutrition, Kansas State College of Agriculture and Applied Science, Manhattan, Kan.

KRAUSS, WILLIAM E., B.S., Ph.D., In Charge of Dairy Nutrition Investigations, Ohio Experiment Station, Wooster, Ohio.

KRISS, MAX, B.S., M.S., Ph.D., Associate Professor of Animal Nutrition, Institute of Animal Nutrition, Pennsylvania State College, State College, Pa.

KYDD, DAVID MITCHELL, B.S., M.D., Associate Physician, Mary Imogene Bassett Hospital, Cooperstown, N. Y.

LAMB, ALVIN R., B.S., M.S., Ph.D., Biochemist, U. S. Public Health Service, U. S. Leprosy Investigation Station, 438 Kuamoo St., Honolulu, Hawaii.

LASHMET, F. H., B.S., M.S., M.D., Magnus Building, Petoskey, Mich.

LEE, MILTON O., B.S., M.A., Ph.D., Associate, The Memorial Foundation for Neuro-Endocrine Research, Harvard Medical School, Boston, Mass.

LEPKOVSKY, SAMUEL, B.S., M.S., Ph.D., Research Associate, Institute of Experimental Biology, University of California, Berkeley, Calif.

LEVINE, HAROLD, B.S., Ph.D., Biochemist, Premier-Pabst Corp., 3821 N. Farwell Ave., Milwaukee, Wisc.

LEVINE, SAMUEL Z., A.B., MD., Professor of Pediatrics, Cornell University Medical College; and Active Pediatrician-in-chief New York Hospital, 525 E. 68th St., New York, N. Y.

LEWIS, HOWARD BISHOP, B.A., Ph.D., Professor and Head of Dept. of Biological Chemistry and Director of College of Pharmacy, Medical School, University of Michigan, Ann Arbor, Mich.

LEWIS, ROBERT C., Ph.D., Professor of Biochemistry, University of Colorado School of Medicine, 4200 E. 9th Ave., Denver, Colo.

LILLY, CORAL A., M.D., Research Fellow in Internal Medicine, University Hospital, University of Michigan, Ann Arbor, Mich.

LYMAN, J. F., B.Sc., Ph.D., Professor of Agricultural Chemistry, Ohio State University, Columbus, Ohio.

MACARTHUR, EDITH H., B.S., A.M., Ph.D., Head of Department of Home Economics, New Hampshire State Normal School, Keene, N. H. (207 Main St.).

MACLEOD, FLORENCE LOUISE, B.S., M.A., Ph.D., Professor of Home Economics, University of Tennessee, Knoxville, Tenn.

MACLEOD, GRACE, M.A., Ph.D., Professor of Nutrition, Teacher's College, Columbia University, New York, 106 Morningside Drive, New York, N. Y.

MACY, ICIE G., A.B., B.S., M.A., Ph.D., Director, Research Laboratory, Children's Fund of Michigan, 660 Frederick St., Detroit, Mich.

MARSH, M. ELIZABETH, M.S., Ph.D., Assistant Professor of Physiology, Department of Vital Economics, University of Rochester Medical School, Rochester, N. Y.

MATTILL, HENRY A., M.A., Ph.D., Professor of Biochemistry, State University of Iowa, Iowa City, Iowa.

MAYNARD, LEONARD A., A.B., Ph.D., Professor of Animal Nutrition, Laboratory of Animal Nutrition, Cornell University, Ithaca, N. Y.

MCCANN, WILLIAM SHARP, A.B., M.D., Dewey Professor of Medicine, Strong Memorial Hospital, 260 Crittenden Blvd., Rochester, N. Y.

MCCAY, C. M., A.B., M.S., Ph.D., Professor of Animal Nutrition, Animal Nutrition Laboratory, Cornell University, Ithaca, N. Y.

MCCLENDON, JESSE FRANCIS, M.S., Ph.D., Professor of Physiological Chemistry, University of Minnesota, Medical School, Minneapolis, Minn., 815 Fulton St., S.E., Minneapolis, Minn.

McCOLLUM, E. V., M.A., Ph.D., Sc.D., Professor of Biochemistry, School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, Md. Member of National Academy of Sciences.

McHENRY, EARLE WILLARD, Ph.D., Assistant Professor Physiological Hygiene, School of Hygiene, University of Toronto, Toronto.

McLESTER, JAMES S., A.B., M.D., LL.D., Professor of Medicine, University of Alabama, 930 S. 20th St., Birmingham, Ala.

McQUARRIE, IRVINE, A.B., Ph.D., M.D., Professor and Head of Dept. of Pediatrics, University of Minnesota, Minneapolis, Minn.

MEIGS, EDWARD B., A.B., M.D., Senior Physiologist, Bureau of Dairy Industry, U. S. Dept. Agriculture, 1736 N St. N.W., Washington, D. C.

MILHORAT, ADE T., A.B., M.D., Research Fellow, Russell Sage Institute of Pathology, N. Y. Hospital, 525 E. 68th St., New York, N. Y.

MILLER, R. C., B.S., Ph.D., Assistant Professor Agricultural and Biological Chemistry, Pennsylvania State College, State College, Pa.

MITCHELL, HAROLD HANSON, M.S., Ph.D., Professor of Animal Nutrition, 555 Old Agriculture Bldg., University of Illinois, Urbana, Ill.

MITCHELL, HELEN S., A.B., Ph.D., Research Professor in Nutrition, Massachusetts State Agricultural College, Amherst, Mass.

MORGAN, AGNES FAY, B.S., M.S., Ph.D., Professor of Household Science, University of California, Berkeley, Calif.

MOULTON, C. ROBERT, B.Sc., M.Sc., Ph.D., Lecturer in the Sciences, Northwestern University, 2253 Sherman Ave., Evanston, Ill.

MUNSELL, HAZEL E., B.A., M.A., Ph.D., Senior Nutrition Chemist in Charge of Nutrition Studies Section, Bureau of Home Economics, U. S. Dept. Agriculture, Washington, D. C.

MURLIN, JOHN R., A.M., Ph.D., Sc.D., Lewis P. Ross Professor of Physiology and Director Department of Vital Economics, University of Rochester Medical School, 260 Crittenden Blvd, Rochester, N. Y.

MYERS, VICTOR CARYL, B.A., M.D., Ph.D., D.Sc., Professor of Biochemistry, Secretary of Medical Faculty, School of Medicine, Western Reserve University, Cleveland, Ohio.

NELSON, ELMER M., B.S., M.S., Ph.D., U. S. Department of Agriculture, Food and Drug Administration, Washington, D. C.

NELSON, P. MABEL, B.S., M.S., Ph.D., Professor and Head of Department of Foods and Nutrition, Iowa State College, Ames, Iowa.

NELSON, VICTOR E., B.S., M.S., Professor of Physiological Chemistry and Nutrition, Chemistry Bldg., Iowa State College, Ames, Iowa.

NEWBURGH, L. H., A.B., M.D., Professor of Clinical Investigation, Dept. of Internal Medicine, University of Michigan, Ann Arbor, Mich.

NORRIS, LEO CHANDLER, B.S., Ph.D., Professor of Poultry Husbandry, Poultry Department, Cornell University, Ithaca, N. Y.

OKEY, RUTH, Ph.D., Associate Professor of Household Science, 1583 Life Science Building, University of California, Berkeley, Calif.

ORTEN, JAMES M., B.S., M.S., Ph.D., Research Fellow, Dept. Physiological Chemistry, Yale University, New Haven, Conn.

OUTHOUSE, JULIA P., B.S., M.S., Ph.D., Associate Professor in Nutrition, Dept. of Home Economics, University of Illinois, Urbana, Ill.

PALMER, LEROY S., B.S., A.M., Ph.D., Professor of Agricultural Biochemistry, Biochemistry Bldg., University Farm, St. Paul, Minnesota.

PARSONS, HELEN T., B.S., M.S., Ph.D., Professor of Nutrition in Home Economics, Home Economics Dept., University of Wisconsin, Madison, Wisc.

PEMBERTON, RALPH, B.S., M.S., M.D., Professor of Medicine, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa. (Mail to Paoli, Pa.).

PETERS, JOHN P., A.B., M.D., John Slade Ely Professor of Internal Medicine, Dept. Internal Medicine, Yale University, 333 Cedar St., New Haven, Conn.

PETERSON, WILLIAM H., B.S., A.M., Ph.D., Professor of Agricultural Chemistry, College of Agriculture, University of Wisconsin, Madison, Wisc.

PIERCE, HAROLD B., B.S., M.S., Ph.D., Junior Professor of Physiological Chemistry, Dept. Vital Economics, University of Rochester Medical School, Crittenden Blvd., Rochester, N. Y.

PITTMAN, MARTHA S., B.S., A.M., Ph.D., Head, Dept. of Food Economics and Nutrition, Kansas State College, Manhattan, Kan.

POLLACK, HERBERT, A.B., M.D., Ph.D., Instructor in Clinical Medicine, Cornell University Medical College, 20 E. 76th St., New York, N. Y.

QUINN, EDMOND J., B.S., Ph.D., Director of Research, The Maltine Company, 106 N. Lee Ave., Rockville Center, Long Island, N. Y.

RABINOWITCH, I. M., M.D., C.W., D.Sc., F.R.C.P., Assistant Professor of Medicine and Lecturer in Biochemistry, McGill University; Director Dept. Metabolism, Montreal General Hospital, Montreal, Que.

RALLI, ELAINE P., A.B., M.D., Assistant Professor of Medicine, New York University College of Medicine, 477 First Ave., New York, N. Y.

REMINGTON, ROE EUGENE, A.B., M.A., Ph.D., Professor of Nutrition, Medical College of South Carolina and Director Laboratory of the South Carolina Food Research Commission, 280 Calhoun St., Charleston, S. C.

RITZMAN, E. G., B.S., A.M. Science (hon.), Research Professor, Durham, N. H.

ROBERTS, LYDIA J., Ph.D., Professor and Chairman, Dept. Home Ecomonics, The University of Chicago, Chicago, Ill.

ROE, JOSEPH H., A.B., A.M., Ph.D., Professor of Biochemistry, George Washington University Medical School, 1335 H St. N.W., Washington, D. C.

ROOT, HOWARD F., A.B., M.D., Assistant Courses for Graduates, Harvard Medical School, 44 Dwight St., Brookline, Mass.

ROSE, ANTON RICHARD, B.S., M.S., Ph.D., Biochemist, Prudential Laboratory, Prudential Insurance Co. of America. Box 376, Edgewater, N. J.

ROSE, MARY SWARTZ, B.S., Ph.D., Professor of Nutrition, Teachers College, Columbia University, New York, N. Y.

ROSE, WILLIAM C., B.S., Ph.D., Professor of Biochemistry, University of Illinois, Urbana, Ill. Member National Academy of Sciences.

ROTH, PAUL, M.D., Director Clinical Laboratories of the Battle Creek Sanitarium Battle Creek, Mich.

ROWNTREE, JENNIE I., B.S., M.S., Ph.D., Professor of Home Economics, University of Washington, Seattle, Wash.

ROWNTREE, LEONARD G., M.S., Sc.D., Director, Philadelphia Institute Medical Research; Research Clinician, Philadelphia General Hospital, 34th and Pine Sts., Philadelphia, Pa.

RUSSELL, WALTER C., Ph.D., Biochemist in Nutrition and Professor of Agricultural Biochemistry, Agricultural Experiment Station, New Brunswick, N. J.

SALMON, W. D., B.S. Agric., A.M., Research Professor in Animal Nutrition, Alabama Polytechnic Institute, Auburn, Ala.

SALTER, WILLIAM THOMAS, A.B., M.D., Research Fellow in Biochemistry, Harvard Cancer Commission, Tutor and Instructor, Biochemical Sciences, Harvard University, Collis P. Huntington Memorial Hospital, 695 Huntington Ave., Boston, Mass.

SAMPSON, MYRA, A.M., Ph.D., Professor and Chairman Department of Zoölogy, Smith College, North Hampton, Mass.

SANDELS, MARGARET R., B.S., A.M., Ph.D., Dean, School of Home Economics, Professor of Nutrition, Florida State College for Women, Tallahassee, Fla.

SANDIFORD, IRENE, A.B., Ph.D., Assistant Professor of Medicine, The University of Chicago, Chicago, Ill.

SANTOS, FRANCISCO O., A.B., M.S., Ph.D., Professor and Head Department Agricultural Chemistry, College of Agriculture, University of Philippines, Los Banos, Laguna, P. I.

SCHARLES, FREDERICK H., M.D., 1405 Bryant Bldg., Kansas City, Mo.

SCHLOSS, OSCAR M., B.S., M.D., Professor of Clinical Pediatrics, Cornell University Medical College, 525 E. 68th St., New York, N. Y.

SCHLUTZ, F. W., A.B., M.D., Chairman of Pediatric Department, Bobs Roberts Memorial Hospital for Children, The University of Chicago, Chicago, Ill.

SEBRELL, W., M.D., Chief of Department of Nutrition, National Institute of Hygiene, Washington, D. C.

SHAFFER, PHILIP A., Ph.D., Professor of Biological Chemistry, Washington University, St. Louis, Mo. Member of the Natonal Academy of Sciences.

SHERMAN, HENRY C., B.S., Sc.D., Ph.D., Mitchill Professor of Chemistry and Executive Officer of Department of Chemistry, Columbia University, New York, N. Y. Member of the National Academy of Sciences.

SHOHL, A. T., A.B., M.D., Research Associate in Pediatrics, Harvard Medical School. 30 Lakeville Rd., Jamaica Plains, Mass.

SMITH, ARTHUR H., M.S., Ph.D., Associate Professor of Physiological Chemistry, Yale University, Sterling Hall of Medicine, 333 Cedar St., New Haven, Conn.

SMITH, MARGARET CAMMACK, A.B., A.M., Ph.D., Professor of Nutrition, University of Arizona; Nutrition Chemist, Agricultural Experiment Station, School of Home Economics, University of Arizona, Tuscon, Ariz.

SOBOTKA, HARRY H., Ph.D., Chemist to Mount Sinai Hospital, Mount Sinai Hospital, 5th Ave. and 100th St., New York, N. Y.

SOSKIN, SAMUEL, M.D., M.A., Ph.D., Director Metabolic and Endocrine Research, Nelson Morris Memorial Institute for Medical Research, Michael Reese Hospital, Chicago; and Assistant Professor of Physiology, The University of Chicago, Chicago, Ill.

SPENCER, HARRY JAMES, A.B., M.A., Ph.D., Associate Professor of Clinical Medicine, Cornell University Medical College; Director Second Medical Division, Bellevue Hospital, New York, N. Y.

SPERRY, WARREN M., M.S., Ph.D., Assistant Professor of Biological Chemistry, Columbia University, Babies Hospital, Broadway and 167th St., New York, N. Y.

SPOHN, ADELAIDE, B.S., M.S., Ph.D., Nutritionist, Elizabeth McCormick Memorial Fund, 848 N. Dearborn St., Chicago, Ill.

STEARNS, GENEVIEVE, B.S., M.S., Ph.D., Research Associate Professor, Pediatrics, State University of Iowa, Iowa City, Iowa.

STEENBOCK, HARRY, M.S., Ph.D., Professor of Agricultural Chemistry, Agricultural Chemistry Bldg., University of Wisconsin, Madison, Wisc.

STIEBELING, HAZEL K., A.B., M.A., Ph.D., Senior Food Economist, Bureau of Home Economics, U. S. Dept. of Agriculture, Washington, D. C.

SUTTON, T. SCOTT, B.Sc., M.Sc., Ph.D., Assistant Professor Ohio State University, Associate Ohio Agricultural Experiment Station, Ohio State University, Columbus, Ohio.

SWANSON, PEARL P., B.S., M.S., Ph.D., Professor Foods and Nutrition, Iowa State College, Ames, Iowa.

SWIFT, RAYMOND W., B.S., M.S., Ph.D., Assistant Professor, Institute of Animal Nutrition, Pennsylvania State College, State College, Pa.

TALBOT, FRITZ BRADLEY, A.B., M.D., Emeritus Professor of Pediatrics, Harvard Medical School; Consultant Massachusetts General Hospital, 270 Commonwealth Ave., Boston, Mass.

TAYLOR, ALONZO E., M.D., Director, Food Research Institute, Stanford Institute, Stanford University, Palo Alto, Calif.

THOMAS, BYRON H., B.S., M.S., Ph.D., Professor and Head Animal Chemistry and Nutrition, Chemistry Section, Iowa Agricultural Experiment Station, Iowa State College, Ames, Iowa.

TILT, JENNIE, B.S., M.S., Ph.D., Professor of Physiological Chemistry and Nutrition, Florida State College for Women, Tallahassee, Fla.

TISDALL, FREDERICK F., M.B., M.D., F.R.C.P.(c), Associate Department of Pediatrics, University of Toronto, Assistant Physician Hospital for Sick Children, Associate Department Medicine, University of Toronto, Toronto, Ont.

TITUS, HARRY W., A.B., A.M., Ph.D., Senior Biological Chemist, Bureau of Animal Industry, U. S. Dept. of Agriculture, 3705–24th St. N.E., Washington D.C.

TOURTELLOTTE, DEE, A.B., M.S., D.Sc., In Charge of Nutrition Laboratory, General Foods Corporation, Battle Creek, Mich.

TRIMBLE, HARRY C., A.B., Ph.D., Assistant Professor of Biological Chemistry, Harvard Medical School, Boston, Mass.

VAHLTEICH, ELLA McCOLLUM, B.S., M.A., Ph.D., 46 Hudson Avenue, Edgewater, N. J.

VARS, HARRY M., A.B., Ph.D., Associate Department Res. Surgery, University of Pennsylvania Medical School, Philadelphia, Pa.

VIGNEAUD, VINCENT DU, B.S., M.S., Ph.D., Professor of Biochemistry, School of Medicine, George Washington University, 1335 H St. N.W., Washington, D. C.

WADDELL, JAMES, B.S.A., M.S., Ph.D., Director Biological Laboratory, E. I. du Pont de Memours & Co., New Brunswick, N. J.

WANG, CHI CHE, B.A., M.S., Ph.D., Research Chemist, Department of Surgery, University of Cincinnati Medical College, Cincinnati, Ohio. 2810 Stratford Ave.

WEBSTER, BRUCE, M.D., C.M., Assistant Professor of Medicine, Cornell University Medical College; Associate Attending Physician N. Y. Hospital, Cornell University Medical College, 1300 York Ave., New York, N. Y.

WHEELER, RUTH, A.B., Ph.D., Professor of Physiology and Nutrition, Vassar College, Poughkeepsie, N. Y.

WHITE, ABRAHAM, B.A., M.A., Ph.D., Instructor in Physiological Chemistry, Yale University, New Haven, Conn.

WILDER, RUSSELL MORSE, B.S., Ph.D., M.D., Professor of Medicine, Mayo Foundation, Rochester, Minn.

WILLIAMS, HAROLD H., B.S., Ph.D., Research Associate, Children's Fund of Michigan, 660 Frederick St., Detroit, Mich.

WINTERS, JET C., B.A., B.S., M.A., Ph.D., Professor of Home Economics, Home Economics Department, University of Texas, Austin, Tex.

WOODS, ELLA, A.M., Ph.D., Home Economist, Experiment Station, University of Idaho, Moscow, Idaho.

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